Universidad Autónoma de Madrid

Facultad de Ciencias

Departamento de Biología Molecular

Programa de Doctorado Biociencias Moleculares

Synthetic dendrimer peptide vaccines against Foot-and-Mouth Disease Virus

Tesis Doctoral

Rodrigo Cañas Arranz

Madrid, 2020

Universidad Autónoma de Madrid

Facultad de Ciencias

Departamento de Biología Molecular

Programa de Doctorado Biociencias Moleculares

Synthetic dendrimer peptide vaccines against Foot-and-Mouth Disease Virus

Tesis Doctoral

Rodrigo Cañas Arranz, Graduado en Biología

Directores de Tesis: Francisco Sobrino Castelló y Esther Blanco Lavilla

Madrid, 2020

Memoria presentada por el Graduado en Biología Rodrigo Cañas Arranz para optar al grado de Doctor en Biociencias Moleculares por la Universidad Autónoma de Madrid.
Madrid, Enero 2020

El trabajo presentado en esta Tesis Doctoral ha sido realizado en el Centro de Biología Molecular "Severo Ochoa", bajo la dirección del Dr. Francisco Sobrino Castelló y la Dra. Esther Blanco Lavilla, y financiado por una beca predoctoral de Formación de Personal Investigador (FPI) del CSIC. Durante esta Tesis Doctoral se realizó una estancia de 97 días en "Icahn School of Medicine at Mount Sinai" (Nueva York, EEUU) en el laboratorio del Dr. Adolfo García Sastre financiada por la beca predoctoral FPI del CSIC.

A los míos

Agradecimientos

Son tantas las personas a las que agradecer su apoyo y cariño que estas líneas se harán cortas. En primer lugar, agradecer a Pachi su confianza desde el primer año que realicé el trabajo de fin de grado, el de máster y ahora la Tesis. De igual manera, agradecer a Esther por co-dirigirme, ayudarme y enseñarme tanto durante estos 4 años.

Gracias a David Andreu y a las fantásticas químicas Mar y Sira, por los péptidos y el trabajo en común que hemos sacado. Sois unos cracks!

A Javier Domínguez y Conchi Revilla (INIA) por las ideas experimentales, el MACS y anticuerpos.

A la gente del laboratorio con los que tan buenos momentos he pasado y tanto cariño me han dado: a Patri, por su grandísimo apoyo y disposición para todo, a Pepa por su incansable cariño y ayuda, a Elisa, por las buenas ratonadas y risas y a Flavia que me enseñó de todo con sus plaqueos. Gracias a todas por los buenos ratos que hemos pasado. Agradecer a Marga su cariño y preocupación por si comía en momentos de estrés. A Miguel por sus buenos consejos y disponibilidad y al nuevo fichaje, Miguel A, por trasmitir siempre alegría.

A la gente del labo que ya no está y con la que inicié mis andanzas en el CBM: Ángela, Mónica, Yuri y en especial a Miguel, que me enseñó todo desde el principio y me animó a no dejar la ciencia. Gracias.

A la gente del INIA y del labo de Juan Carlos: Estela, Ana y Nereida, por los geniales momentos y copas tomadas en congresos (y no congresos).

A la gente de cultivos: Raquel, Elena y Dani por su buen rollo, qué majos sois. A Alicia, Silvia, Ana e Isa por las charlas y risas entre infección e infección. A Alberto, por ayudarme tanto con los papeles de la visa. A Andrea y Cris, por ser tan amigables y cercanas.

A las personas encargadas de cultivos (Nunci y Mª Ángeles) a las chicas de citometría (Berta, Raquel y Silvia), al personal de Animalario del CBM y del Departamento de Reproducción de INIA (Juan Carlos y Andrés). Sin el trabajo de todos ellos no habría sido posible.

I would like to thank Adolfo for having me at his lab during my stay at MSSM. Also all his lab, specially Michael, who helped me a lot and took care of me the time I spent in NY. A little piece of this Thesis is yours as well. And the rest! Sara, Maite, Angela, Ryan, Randy, Paula…y sobre todo a Elena, a la que tanto coñazo he dado con papeles y demás, y a la que le debo en la mayor parte haber pasado una experiencia inolvidable en NY (aunque en Las Vegas no jugó ni un dólar a la ruleta).

Quiero agradecérselo también a mi compañero de fatigas, Briso, con el que nos hemos desahogado en momentos de estrés y a Esme, por el cafecito de las 5 que tan bien nos sentaba y a que eventualmente se unía El Cántabro. Y al equipo de fútbol del CBM!

A Sara, Mateo y Guille a los que no les queda nada…mucho ánimo! A Raúl, mi compi de piso de NY que tantas risas nos echamos y tan a gusto estuvimos conviviendo. A Javi, Alex, Diana, Rafita, Alfredo y Carlos que les tengo abandonados, pero espero que no dure mucho. Al Cuis, que, aunque no pesque nada y encaje goles a mansalva en el "fifi", se le quiere y es un fenómeno. A

A los biologucos! Jacobo, Fernando, Karima, Mónica, Laura, Nerea, Inés y María, que, aunque alguno esté lejos, el cariño se siente muy cerca. Esta Tesis es vuestra! por vosotros!

Por último, a los cercanos: mis primos (alguno más pesado que otro) y los de casa: mi madre Mª Carmen, mi padre Jesús y mi hermano Álvaro. Sin su apoyo nada de esto hubiera sido posible. Va por vosotros. Un beso enorme a los 3.

PS: También a los 635 ratones y lactantes que pasaron por mis manos y las 56 cerditas que dieron lugar a esta Tesis.

Abbreviations

ANOVA	Analysis Of Variance
APC	Antigen Presenting Cell
BEI	Binary Ethylenimine
BFA	Brefeldin A
BHK	Baby Hamster Kidney (cell line)
BSL	Biosafety Level
CBMSO	Centro de Biología Molecular Severo Ochoa
cDNA	Complementary DNA
CIB	Centro Investigaciones Biológicas
CSIC	Consejo Superior de Investigaciones Científicas
Da	Dalton
DC	Dendritic Cell
DEPC	Diethyl pyrocarbonate
DIVA	Differentiating Infected from Vaccinated Animal
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
e.g	Exempli gratia (latin). It means "for example"
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent SPOT
ER	Endoplasmic Reticulum
ERAP1	Endoplasmic Reticulum Aminopeptidase 1
EU	European Union
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FMD	Foot-and-Mouth Disease
FMDV	Foot-and-Mouth Disease Virus
g	gravity acceleration

h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IBRS-2	Instituto Biologico-Rim Suino-2 (cell line)
i.e	Id est (latin). It means “this is”
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukine
INIA	Instituto Nacional de Investigación Agraria y Alimentaria
mAb	monoclonal Antibody
MACS	Magnetic Activated Cell Sorting
MHC	Major Histocompatibility Complex
min	minute
ml	milliliter
MOI	Multiplicity Of Infection
nAb	neutralizing Antibody
NCR	Non-coding Regions
NK	Natural Killer
NSP	Non Structural Protein
nt	nucleotide
OIE	Office Internationale des Epizooties
ORF	Open Reading Frame
p	p-value
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
pc	post-challenge
PFA	Paraformaldehyde
PFU	Plaque Forming Unit
PHA-M	Phytohemagglutinnin-M
pi	post-immunization

RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium
RTqPCR	Retrotranscription quantitative Polymerase Chain Reaction
RT	Room Temperature
s	second
S	Svedberg
scFv	Single-chain Variable Fragment
SLA	Swine Leukocyte Antigen
TAP	Transporter associated with antigen processing
T_c	T cytotoxic cell
TCID	Tissue Culture Infective Dose
TCR	T-cell Receptor
T_h	T helper cell
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
UTR	Untranslated Region
µl	microliter
VNT	Viral Neutralizing Titer
VRC	Viral RNA Copies

One and three letter code for amino acids

Alanine	Ala, A	Leucine	Leu, L
Arginine	Arg, R	Lysine	Lys, K
Aspartic acid	Asp, D	Metionine	Met, M
Asparagine	Asn, N	Phenylalanine	Phe, F
Cysteine	Cys, C	Proline	Pro, P
Glutamic acid	Glu, E	Serine	Ser, S
Glycine	Gly, G	Tyrosine	Tyr, Y
Glutamine	Gln, Q	Threonine	Thr, T
Histidine	His, H	Tryptophan	Trp, W
Isoleucine	Ile, I	Valine	Val, V

Index

Summary

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals that is produced by a virus belonging *Picornaviridae* family: FMD virus (FMDV). Although the mortality rate is low, FMD is very feared in farm industry and animal health since in case of an outbreak, massive culling of infected or suspected animals is carried out, causing a fatal economic impact. Current vaccines based on inactivated viruses are being used for disease control in endemic countries. Nevertheless, the several drawbacks of these vaccines, have led the FMD-free countries to apply non-vaccination policies, increasing the risk of disease reintroduction and severe outbreaks. Therefore, the development of safer and effective vaccines is a major priority for FMD control. Synthetic dendrimer peptides are among the different strategies to develop new FMD vaccines. Our laboratory, is using a dendrimer peptide, termed B_2T-3A, as a vaccine model. B_2T-3A harbors two copies of the major antigenic B-cell site [VP1 (140-158)], which is the main target for neutralizing antibodies (nAb), covalently linked to a promiscuous and heterotypic T-helper epitope from the non-structural protein 3A [3A (21-35)]. The administration of two doses of 2 mg of B_2T-3A, protects pigs from FMDV experimental challenge. Interestingly, the modular design of this dendrimer peptide allows modifications aimed at improving its immunogenicity and its vaccine performance, some of which have been explored in this Thesis, as detailed below.

i) The immunogenicity of dendrimer peptides derived from B_2T-3A either synthesized with a different chemistry or harboring different FMDV T-cell epitopes previously identified in pig, have been characterized. The peptide B_2T-TB_2 –displaying two B_2T-3A molecules covalently linked tail-to-tail- induced the highest nAb titers. For this reason, the immunogenicity of this construction was analyzed in pig.

ii) The immunization of pigs with a single dose of B_2T-TB_2 or B_2T-3A, induced high levels of IgG1 and IgG2 specific antibodies along nAb. Also, a strong and specific response of T-cells expressing IFN-γ, the main pro-inflammatory cytokine, was detected upon *in vitro* recall of peripheral blood mononuclear cells (PBMCs). After the boost, nAb and IFN-γ-producing cells could be detected up to 5 months post-immunization.

iii) One single dose of B_2T-3A, of 2 mg or 0.5 mg, protects pigs upon homologous FMDV experimental challenge.

iv) The dendrimer peptide B_2T-3D, harboring a T-helper epitope from FMDV 3D protein [3D (56-70)], and dendrimers with both T-cell epitopes covalently link in tandem in both orientations (B_2T-3A3D and B_2T-3D3A), were selected to study their immunogenicity and the duration of the immune response in pigs. B_2T-3D induced nAb titers and IFN-γ-producing cells similar to those of B_2T-3A. The peptides harboring a copy of each of the T-cell epitopes (B_2T-3A3D and B_2T-3D3A) elicited a strong response of IFN-γ-producing cells, mainly due to the epitope T-3D, suggesting that this epitope is immunodominant over T-3A.

v) IFN-γ-producing cells activated by these peptides were, mainly, $CD4^+$ T-cells although a minor contribution of $CD8^+$ T-cells was as well observed.

vi) The majority of $CD4^+$IFN-γ^+ cells showed a memory ($CD4^+2E3^-$) and a multifunctional phenotype, as they expressed IFN-γ and TNF-α, suggesting that the peptides induced a potent Th1 pro-inflammatory response.

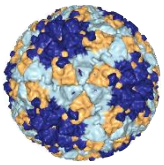
All-in-all, these results strengthen the value and future use of synthetic dendrimer peptides as alternative FMDV vaccines.

Resumen

La fiebre aftosa (FA) es una enfermedad altamente contagiosa que afecta a animales domésticos y salvajes de pezuña hendida y que está producida por un virus de la familia *Picornaviridae*: el virus de la FA (VFA). Aunque su tasa de mortalidad es baja, la FA es una enfermedad muy temida en el sector ganadero, ya que cuando aparece puede originar el sacrificio de innumerables animales afectados o sospechosos de serlo, originando un fuerte impacto económico. En la actualidad, la vacunación con virus inactivado es la mejor medida para controlar la enfermedad en países donde es endémica. Sin embargo, estas vacunas, tiene una serie de desventajas que han llevado a los países desarrollados a aplicar políticas de no vacunación, lo que aumenta el riesgo de la introducción de la enfermedad en estas zonas. Por ello, el desarrollo de nuevas vacunas eficaces y seguras es una prioridad en la lucha contra esta enfermedad. Dentro de las diferentes estrategias para el desarrollo de nuevas vacunas frente a FA se encuentran los péptidos sintéticos. Nuestro laboratorio utiliza como modelo un péptido dendrimérico, B_2T-3A, que contiene dos copias del epítopo B [VP1 (140-158)] principal sitio antigénico de la cápsida de VFA y diana de anticuerpos neutralizantes, unido covalentemente a un epítopo T cooperador, promiscuo y heterotípico localizado en la proteína no-estructural 3A [3A (21-35)]. La administración de dos dosis de 2 mg de B_2T-3A a cerdos los protege frente a un desafío experimental con VFA.

Los estudios realizados se pueden resumir como se indica: i) Se ha caracterizado la inmunogenicidad en ratón, elegido como modelo de *screening* de péptidos análogos a B_2T-3A, sintetizados con estrategias químicas complementarias, que incluyen diferentes epítopos T previamente definidos en cerdo. El péptido B_2T-TB_2 – dos moléculas de B_2T-3A unidas covalentemente cola-con-cola, es el que mayores niveles de anticuerpos neutralizantes (AcNs) indujo. Por ello, se decidió continuar su análisis en cerdo. ii) La vacunación de cerdos con una única dosis de B_2T-TB_2 o de B_2T-3A indujo altos títulos de anticuerpos específicos tanto IgG totales, IgG1 e IgG2, como AcNs, así como una potente estimulación de linfocitos que expresaban la citoquina pro-inflamatoria IFN-γ. En animales revacunados, se pudieron detectar tanto AcNs como células productoras de IFN-γ hasta 5 meses tras la primera inmunización. iii) Se ha determinado que una única dosis de B_2T-3A, tanto de 2 mg como de 0.5 mg, confiere protección sólida en cerdos. iv) el péptido B_2T-3D, que contiene un epítopo T definido como cooperador en la proteína 3D [3D (56-70)] con criterios similares a los utilizados para [3A (21-35)], y los péptidos que incluyen estos dos epítopos T unidos en tándem en ambas orientaciones (B_2T-3A3D y B_2T-3D3A) fueron seleccionados para analizar su inmunogenicidad y la duración de la respuesta específica que inducían en cerdo. El péptido B_2T-3D indujo títulos de AcNs y de células productoras de IFN-γ similares a los de B_2T-3A. En los péptidos conteniendo los epítopos T en tándem (B_2T-3A3D y B_2T-3D3A) se observó, también, una potente inducción de células productoras de IFN-γ, debida, principalmente, al péptido T-3D, sugiriendo que este epítopo es inmunodominante sobre T-3A. v) Las células productoras de IFN-γ inducidas por estos péptidos fueron, mayoritariamente linfocitos T $CD4^+$, observándose también una menor contribución de linfocitos T $CD8^+$. vi) La mayoría de células $CD4^+$ que producían IFN-γ^+ mostraban un fenotipo de memoria ($CD4^+2E3^-$) y, presentaban un carácter multifuncional, ya que en su mayoría secretaban conjuntamente IFN-γ y TNF-α, sugiriendo que estos dendrímeros inducen una potente respuesta pro-inflamatoria Th1.

En conjunto, estos resultados apoyan el uso de los péptidos dendriméricos como una alternativa vacunal viable contra VFA.



INTRODUCTION

2. Introduction

2.1. The disease: Overview and pathogenesis

Foot-and-mouth disease (FMD) is an acute and systemic vesicular disease that affects artiodactyls (cloven-hoofed) farm animals such as cattle, pigs, goats, sheep, water buffaloes, alpacas, llamas, bactrian camels and yacks, as well as more than 70 wild ruminant species (Nfon et al., 2017). The basis of this wide host-range remains unknown and is one of the most challenging aspects of the biology of the etiological agent of this disease: FMD virus (FMDV).

A disease that is considered as FMD was first described in Venice in 1546 (Fracastoius, 1546). In 1898, Loeffler and Frosch demonstrated that a filterable infectious agent smaller than bacteria caused FMD; this was the first description of a virus producing an animal disease (Loeffler and Frosch, 1897). Since then, despite the large amount of information gained on the virus and the disease, as well as the availability of vaccines, FMD still affects wide areas of the world (**Figure 1**). Although the rates of mortality caused by FMD are low, the disease is highly transmissible and morbidity can reach 100%. The fatal impact of FMD outbreaks is resumed in severe decreases in livestock production, which contributes to countless economic losses in many developing countries and devastating economic aftermath (Pereira, 1981; Sobrino and Domingo, 2001). Indeed, FMD is included in the list of notifiable terrestrial and aquatic animal diseases of the World Organization for Animal Health (OIE) −formerly the Office Internationale des Epizooties −, and is responsible for important trade restrictions on animals and livestock products. Examples of the devastating FMD epizootics over the past decades are those occurred in Taiwan (1997) (Kitching, 1998), Argentina (2000-2001) (Mattion et al., 2004), UK (2001-2002) (Knowles and Samuel, 2003), China (2005), Japan (2010-2011) and South Korea (2010-2014) (Brito et al., 2017). In each of these epizootics million animals were slaughtered and the estimated losses were very high.

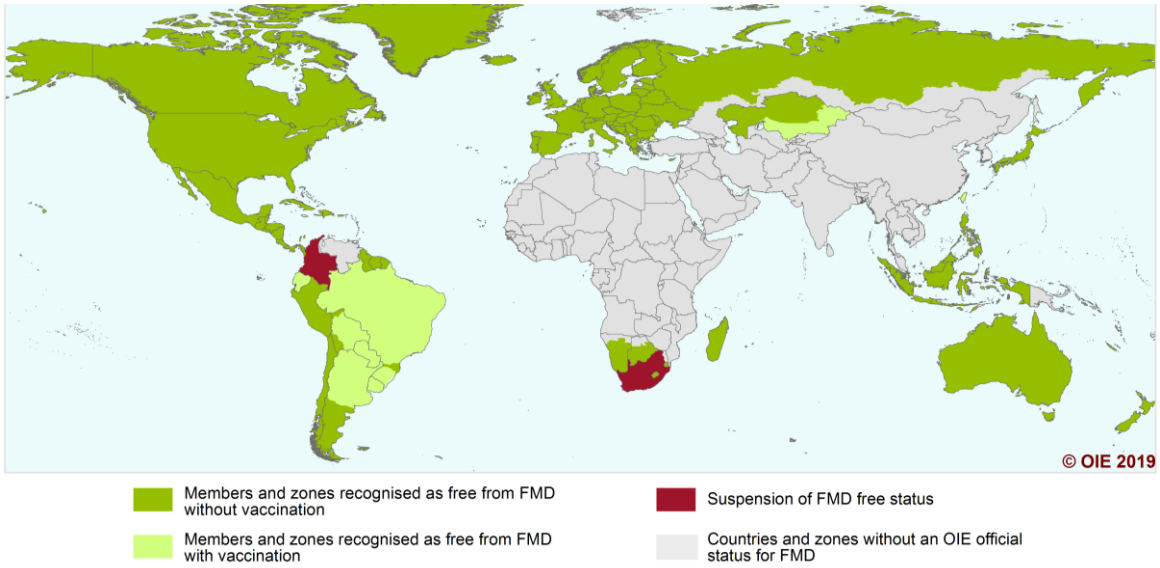


Figure 1: Overview of the current epidemiological status of FMD worldwide. Each country/zone is colored as follows: FMD-free without vaccination (dark green), FMD-free with vaccination (light green), suspension of FMD free status (red), without official status for FMD, associated with FMD endemic countries (grey). Adapted from OIE in May 2019 (https://www.oie.int/en/animal-health-in-the-world/official-disease-status/fmd/en-fmd-carte/).

In natural infections, the main route of FMDV entry is the upper respiratory tract. Nevertheless, the susceptibility and primary route of infection may depend on the species, being cattle more susceptible by aerosol while pigs are more prone to be primary infected by skin abrasions (Alexandersen and Mowat, 2005; Alexandersen et al., 2003; Sellers and Gloster, 2008). Within 24-48 h after infection, fever and viremia start along with the virus entry in the blood stream and its spread to different tissues and organs, producing secondary vesicles preferentially in the mouth and feet. FMD clinical signs include fever, salivating, loss of appetite, lameness and occasionally mastitis (Nfon et al., 2017). FMDV can be mechanically disseminated by animals, farmers, farming equipment, and during animal transport (Brooksby, 1982) and airborne transmission to long distances has been reported (King et al., 1981). It has been proposed that macrophages may contribute to the viral spread throughout the body observed during the viremia, although the mechanisms behind virus dissemination in infected animals are poorly known (Baxt and Mason, 1995). The acute phase of disease lasts about one week and declines gradually, concomitantly with the induction of a strong antibody response. Secondary bacterial infections in foot vesicles can result in chronic lameness, wasting and, eventually, death. Mortality in adults is relatively low but it can become high in young animals in a process associated to acute viral myocarditis (Gulbahar et al., 2007).

A persistent, asymptomatic infection can occur in ruminants (Arzt et al., 2019; Bronsvoort et al., 2016) during which the virus is eventually isolated from the esophagus and throat fluids from infected animals beginning a few weeks up to several years of the initial infection (Salt, 1993). Both naive and vaccinated cattle can become persistently infected following an acute infection (Alexandersen et al., 2002; Moonen and Schrijver, 2000; Sutmoller and Gaggero, 1965; Vosloo and Thomson 2017). In buffalo, virus disappears upon two weeks post infection from secretions of all organs but the pharynx, where low-level of viral replication can be detected in a substantial number of animals, rendering buffalo as a possible carriers in FMD outbreaks in South Africa (Hedger and Condy, 1985; Vosloo and Thomson 2017). In fact, it has been reported that persistently infected animals may be at the origin of FMD outbreaks, when brought into contact with susceptible animals (Hedger and Condy, 1985), although its epidemiological significance is still controversial. The mechanisms that mediate FMDV persistence remain unclear, but it has been proposed that in carrier animals a dynamic equilibrium between the host immune response and the selection of viral antigenic variants at the mucosae of the upper respiratory tract is established (Biswal et al., 2019; Cortey et al., 2019; Gebauer et al., 1988; Stenfeldt et al., 2017). In addition, the inhibition of apoptosis and-cell mediated immunity in the nasopharynx (Eschbaumer et al., 2016), as well as the suppression of antiviral host factors *i.e.* gamma interferon (IFN-γ) and lambda interferon have been suggested to contribute to FMDV persistence in cattle (Stenfeldt et al., 2016).

2.2. The virus: genomic organisation and cell attachment

FMDV is a positive-sense, single-stranded RNA virus that belongs to the *Aphthovirus* genus, within the *Picornaviridae* family. As occurred in RNA virus, FMDV populations are genetically and antigenically diverse and this is reflected in the existence of seven serotypes: A, O, C, SAT-1, 2 and 3 as well as multiple variants within each serotype [for revisions, see (Grubman and Baxt, 2004; Mason et al., 2003; Sobrino et al., 2001).

The FMDV particle is composed of a non-enveloped icosahedral protein capsid (140S particle) that surrounds a single stranded positive-sense viral RNA molecule of about 8.500 nt in length. The genomic RNA encompasses a single open reading frame (ORF) flanked by two highly structured non-coding untranslated regions (UTRs) that contain key structural elements for viral replication and gene expression (**Figure 2**) (Belsham, 2005; Saiz et al., 2001).

While most cellular mRNAs exhibit a cap structure at their 5' end, this element is absent at the 5' UTR of FMDV RNA that harbors an internal ribosome entry site (IRES) element that promotes the cap-independent translation initiation of the viral genome.

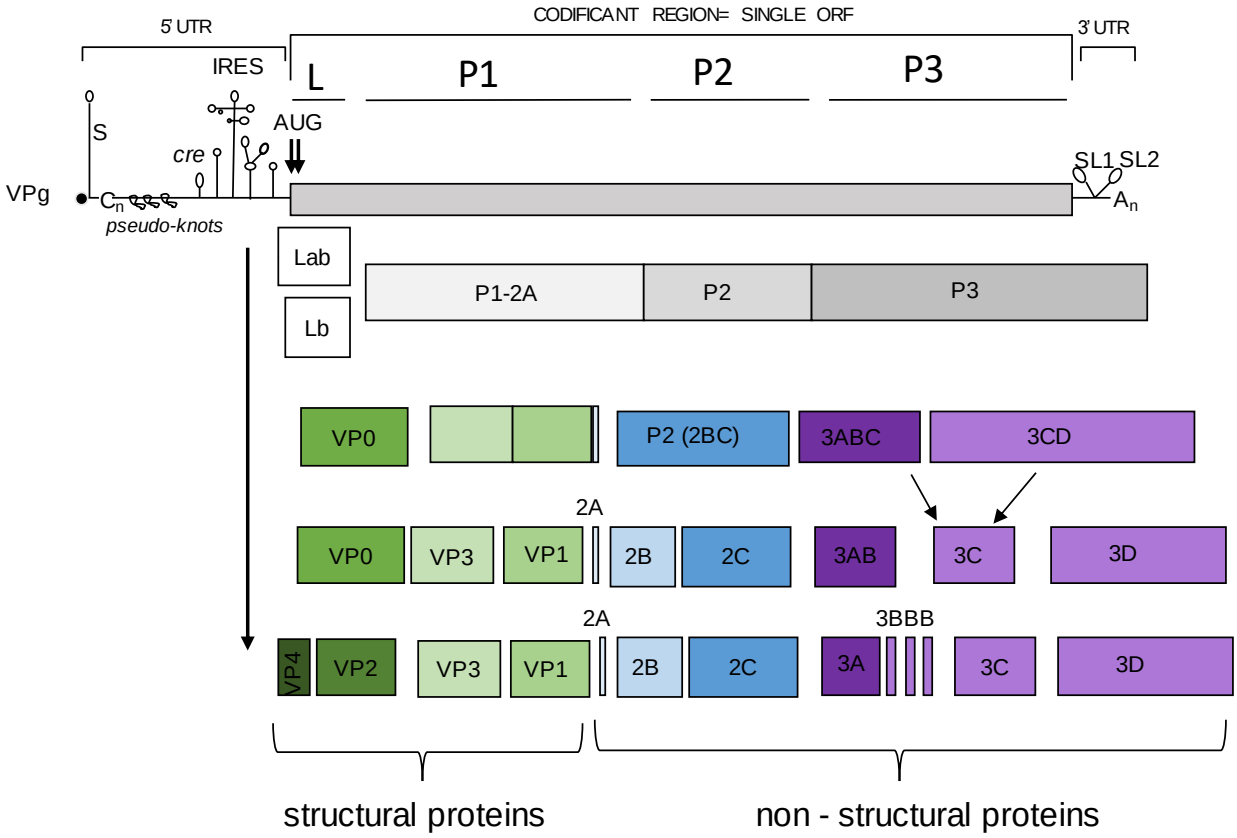


Figure 2: Genomic organization of FMDV. On top, a diagram of functional regions in the viral RNA molecule. The ORF, boxed in grey, is flanked upstream and downstream by non-coding regions (NCR) important for viral replication. The precursors and mature proteins derived from the processing of the polyprotein by viral proteases, are depicted in color boxes. Structural proteins are colored in green and non-structural protein in blue or purple. Adapted from (Martin-Acebes, 2009).

FMDV RNA is polyadenylated at its 3'end and contains a small viral-encoded protein, 3B, also termed VPg, covalently linked to its 5'end (**Figure 2**) (Andreev et al., 2007; Belsham, 1992, 2005). Genomic RNA is translated into the viral polyprotein from two in-frame AUG codons, being the second one preferentially used to synthesize, a polyprotein of about 250 kDa, which is subsequently cleaved by the viral proteases Leader (L^{pro}) and $3C^{pro}$ to yield structural and non-structural proteins (NSPs). The FMDV ORF can be divided into four functional areas based on the location of the primary cleavages and the functions of mature encoded polypeptides (Grubman and Baxt, 2004): (i) L region, which is located at 5′ end to ORF and codes for two alternative forms of L^{pro} termed L_{ab} and L_b, (ii) The P1 region, encoding the precursor for capsid polypeptides, which can generate four mature capsid proteins: 1A, AB, AC and 1D, also termed VP4,

VP2, VP3 and VP1, respectively, (iii) The P2 region that encodes three viral proteins: 2A, 2B, and 2C and (iv) The P3 region that encodes four viral proteins: 3A, 3B, $3C^{pro}$ and $3D^{pol}$, among which, 3C is a viral protease and 3D an RNA-dependent RNA polymerase (**Figure 2**).

The virus initiates the infection by recptor-mediated binding to the host cell. FMDV uses integrins such us $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_8$ as receptors, being $\alpha_v\beta_6$, an integrin widely expressed in epithelia cells, the main target for viral replication *in vivo* (Duque and Baxt, 2003; Jackson et al., 2000; Lawrence and Rieder, 2017). Binding of the viral particle to its receptors occurs through a conserved triplet in VP1 capsid protein: the RGD motif (Acharya et al., 1989; Jackson et al., 2003). This region is essential for FMDV biology since it functions as receptor binding site as well as the main target for virus neutralizing antibodies (nAbs) (see below). This motif is located in the so-called G-H loop that spans residues 133-158 of VP1capsid protein. Although this RGD triplet is conserved, the G-H loop is highly variable among serotypes and also shows intra-serotype variation, since it is part of the main B-lymphocyte (B-cell) antigenic site of the virus (site A). Alternatively, culture-adapted FMDV can bind cells in a RGD-independent manner by using proteoglycan present in the extracellular matrix such as heparan sulfate (HS). The acquisition of the capacity to recognize HS as receptor has been directly related to the selection of positively charged amino acid residues on the viral capsid that would interact with the negative charges of HS (Baranowski et al., 1998; Jackson et al., 1996; Sa-Carvalho et al., 1997). However, HS-adapted viruses are attenuated *in vivo*, which supports that HS is not used as a receptor in natural hosts (Sa-Carvalho et al., 1997). It has been recently suggested that FMDV can also exploit macropynocitosis to gain entry into the cell (Han et al., 2016).

2.3. The structure and antigenicity of the virion

2.3.1 Structure of the capsid

FMDV was one of the first animal viruses for which the three-dimensional (3D) structure of its particle was resolved (Acharya et al., 1989; Lea et al., 1994). In general, FMDV virions share broad structural similarities with those of other picornaviruses (Fry et al., 2005; Han et al., 2015; Kotecha et al., 2017). The small FMD virion (30 nm in diameter) is roughly spherical and is composed of a nonenveloped icosahedral capsid with a smooth surface that accommodates a single molecule of the RNA genome (**Figure 3**) (Mateu, 2017). The mature FMDV capsid is made of 60 copies of each of three major structural proteins VP1, VP2, VP3 (each of about 210 –220 residues in length) that are exposed in the particle surface. VP4 is a smaller (about 80 residues), internal capsid protein that is

myristoylated at its N-terminus. Indeed, VP4 can be considered as a long N-terminal extension of VP2 that is released by proteolytic cleavage during virion maturation (**Figure 3**) (Chow et al., 1987).

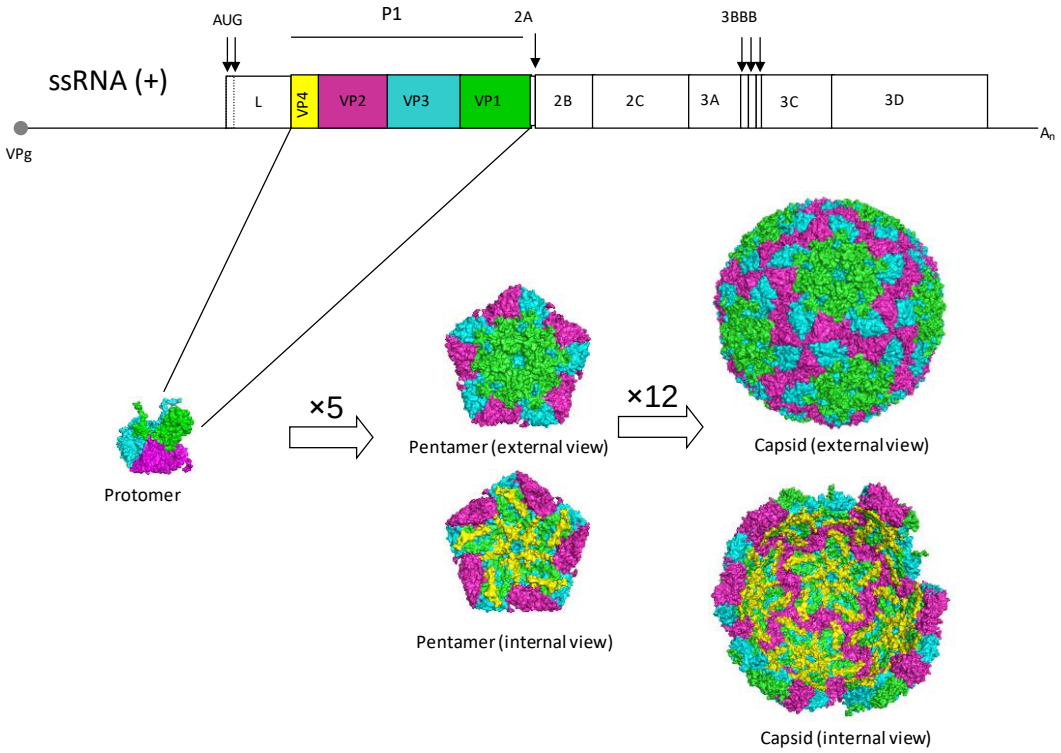


Figure 3: Structural proteins and capsid assembly. The top panel represents the genomic viral RNA molecule linked to VPg at its 5' end and to a polyA tail (An) at its 3' end. The colored boxes indicate the structural proteins VP4 (yellow), VP2 (purple), VP3 (cyan) and VP1 (green). The panel below represents the intermediates for capsid formation. One copy of each structural protein conforms the biological protomer. The assembly of 5 protomers originates the pentamer and 12 pentamers assemble to form the capsid (Lea et al., 1994). For a detailed explanation see the text. Adapted from (Martin-Acebes, 2009).

The structurally similar VP1, VP2 and VP3 display a core containing eight β-strands that form two four-stranded β-sheets and are connected by loops of variable length, each denoted according to the two strands they connect (Acharya et al., 1989). These surface-exposed loops contribute to the immunogenic and antigenic characteristics of the virus particle (Curry et al., 1995; Lea et al., 1994; Mateu and Verdaguer, 2004; Parry et al., 1990), as well as in the case of the GH-loop to the binding to the cell receptor (Fox et al., 1989).

A single copy of each of the capsid proteins associate through non covalent interactions to generate 60 equivalent, roughly trapezoidal structures, the biological protomers from which the capsid is assembled. Five protomers associate around each capsid five-fold axis to form a higher-order pentagonal capsid substructure termed the pentamer. The protomers in each pentamer are held together by multiple non-covalent interactions that

are weaker than those that stabilize the intraprotomer interactions. In addition, the association of the N-termini of VP3 and VP4 around each of the five-fold axis contributes to connect the protomers in the pentamer. Capsids are finally made up of twelve pentamers that are the functional intermediates of capsid assembly and disassembly (**Figure 3**).

The interface between adjacent pentamers is mainly stabilized by electrostatic interactions among VP2-VP3 and VP2-VP2 residues at opposites pentamers, as well as by hydrogen bridges and weak hydrophobic interactions (Acharya et al., 1989; Lea et al., 1994; Mateo et al., 2003). FMDV capsid is dissociated into 12S pentameric subunits at pH below 6.5 (Brown and Cartwright, 1961), a unique feature among picornaviruses. It has been proposed that this high acidic sensitivity is due to the contribution of a cluster of His residues located at the pentamer interface that become protonated at low pH, weakening the capsid stability by electrostatic repulsion (Curry et al., 1995).

2.3.2 Antigenic structure

The antigenic sites recognized by B-cells to produce neutralizing FMDV antibodies are composed of amino acid residues exposed on the surface of the capsid (Acharya et al., 1989). In serotypes A, O, C, Asia 1 and SAT 2, a major continuous (conformation independent) antigenic site is located at the surface exposed G-H loop that connects the βG and βH strands of capsid protein VP1 (Bittle et al., 1982; Grazioli et al., 2013; Opperman et al., 2012; Pfaff et al., 1982; Strohmaier et al., 1982) (see **Figure 4**). This region, which is differently denoted depending on the serotype − site A for serotype C and site 1 for serotype O−, has been proposed as a major antigenic site eliciting antibody protective responses (Acharya et al., 1989; Kotecha et al., 2017; Lea et al., 1994). Indeed, a large proportion of monoclonal antibody (mAb) resistant mutants obtained using mAbs raised against entire FMDV particles showed amino acid substitutions within this site (Baranowski et al., 1998; Mateu, 1995). A detailed study with FMDV isolates of serotype C revealed a complex antigenic structure for the G-H loop, since different overlapping epitopes, defined by their differential ability to react with individual mAbs, were identified (Mateu et al., 1990). An additional neutralisation antigenic site, identified at the C-terminus of VP1, is apparently continuous and independent from the G-H loop in serotypes A and C (Lea et al., 1994). In the capsid of viruses of serotype O, the C-terminus of VP1 appears in close proximity to the G-H loop of this protein, and competition studies with nAbs suggest that both VP1 regions conform a single antigenic site composed of discontinuous epitopes (Barnett et al., 1989). In addition, two new neutralizing epitopes

involving VP3 residues have been identified recently in serotype O although its relevance in protective immunity remains unclear (Mahapatra et al., 2019).

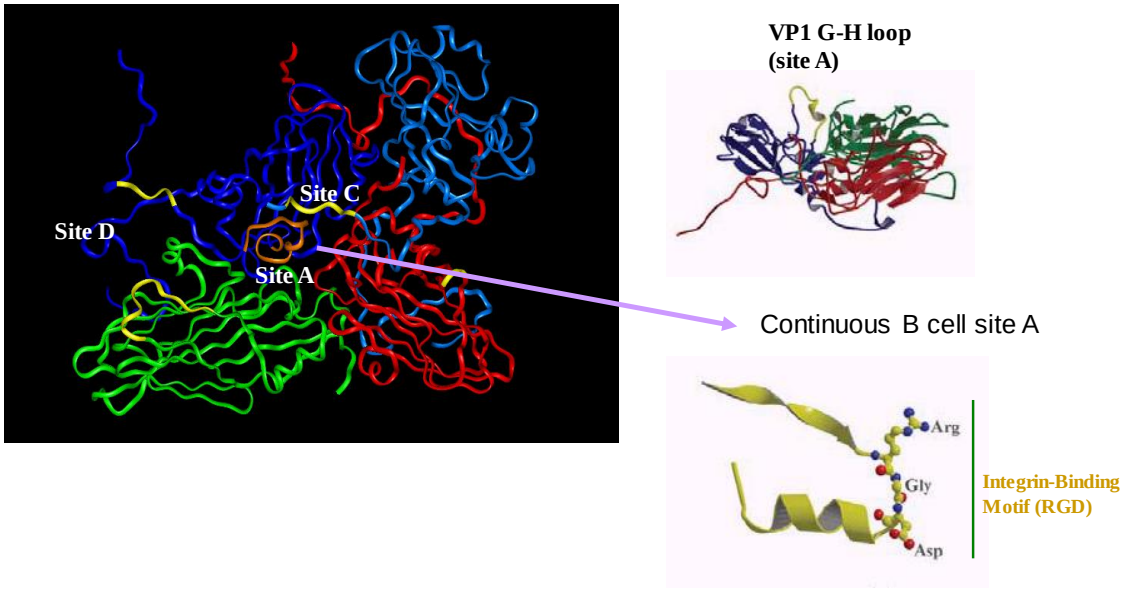


Figure 4: Representation of the B-cell antigenic sites on the capsid of FMDV type C. On the right: details of the VP1 G-H loop (up) and of the RGD motif that mediates virus binding to integrin (cell receptor) (down) are depicted. Adapted from (Mateu et al., 1994).

In serotypes A, O and Asia 1, cross-neutralization studies using FMDV mutants resistant to different antibodies led to the identification of discontinuous antigenic sites. These discontinuous sites are conformed by residues from different capsid proteins (Baxt et al., 1989; Grazioli et al., 2013; Kitson et al., 1990), and are located at exposed regions adjacent to each other and close to the 3-fold axis of symmetry of the capsid. In serotype C, the discontinuous site D comprises residues involving the C-terminus of VP1, the VP3 B-B knob, and VP2 B-C loop (**Figure 4**) (Lea et al., 1994).

2.4 Adaptive immune response against FMDV

Adaptive immunity leading to protection against FMD relies on the activation of T-lymphocytes (T-cell immunity) and the production of antibodies (humoral immunity) (McCullough and Sobrino, 2004). Antigen processing and presentation in the context of MHC class II molecules of T-cell epitopes from viral proteins allow their recognition by T-helper (Th)-lymphocytes (CD4 T-cells). This process triggers the production by T-cells of cytokines such as IFN-γ and differentiation factors, as well as of cognate interactions with B- cells, all required for the development and maturation of the immune response (**Figure 5**) (McCullough and Sobrino, 2004; Robinson et al., 2016). The interaction of FMDV with antigen presenting cells (APCs) is considered the initial step for mounting

efficient T-cell responses. Indeed, uptake of viral antigens by APCs such us dendritic cells (DCs) is particularly relevant, as DCs are key controllers of immune defence development and responsiveness, providing essential antigen presentation to T-lymphocytes and antigen delivery to B-lymphocytes in lymphoid tissues (**Figure 5**) (McCullough et al., 2017). It has been reported that FMDV can interact with APCs and DCs resulting in a sort of abortive infection, and that this process is enhanced when the virus is complexed to specific antibodies (McCullough et al., 1988).

Once the FMDV antigen is processed into small peptides, MHC-II molecules can bind viral epitopes in the so-called MHC-II-rich compartment (MIIC) giving rise to peptide-loaded MHC-II molecules, which are transferred to the cell surface. These MHC-II/peptide complexes are then presented by APCs to the T-cell receptor (TCR) on Th cells (CD4 T-cells). Then, activated CD4 T-cells secrete proinflamatory cytokines to "help" both B-cells and T-cytotoxic (Tc) lymphocytes (CD8 T-cells) to clear the infection.

MHC-I molecules from FMDV-infected cells can also recognized viral peptides that are transported to the cell surface and recognized by the TCR on CD8 T-cells, which kill the infected cells, avoiding subsequent multiplication of the virus. For MHC-I presentation, viral proteins are degraded in the cytosol via proteasome and then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). Within the ER, the MHC-I/peptide complexes are formed and then transported to the Golgi and the cell surface for later presentation to the TCR on CD8 T-cells. MHC-I presentation is not restricted to FMDV-infected cells, since a cross-presentation phenomenon allows the activation of CD8 T-cell by APCs. Nevertheless, a clear picture on the role of CD8 T-cells in the outcome of the protective response against this virus is not yet available (see below).

B-lymphocytes (B-cells) are able to recognize FMDV antigens and produce specific antibodies against them. However, optimal antibody production depends on the interaction with CD4 T-cells, which expresses cytokines that drive B-cells towards professional antibody-producing plasma cells. Once antibodies are synthesized, they can bind directly to the virus, inhibiting their attachment to susceptible cells, or being recognized by phagocytes through their Fc fragments of the Fc receptors (FcR), forming the so called antigen opsonized complexes to be internalized and degraded (McCullough and Sobrino, 2004).

Altogether, the initial adaptive immune response contributes to clear the viral infection and leads to the so called immune memory exerted by activated B and T-cells that is on

the basis of vaccine development. A generalised overview showing the key points of FMDV adaptive immune response is shown in **Figure 5**.

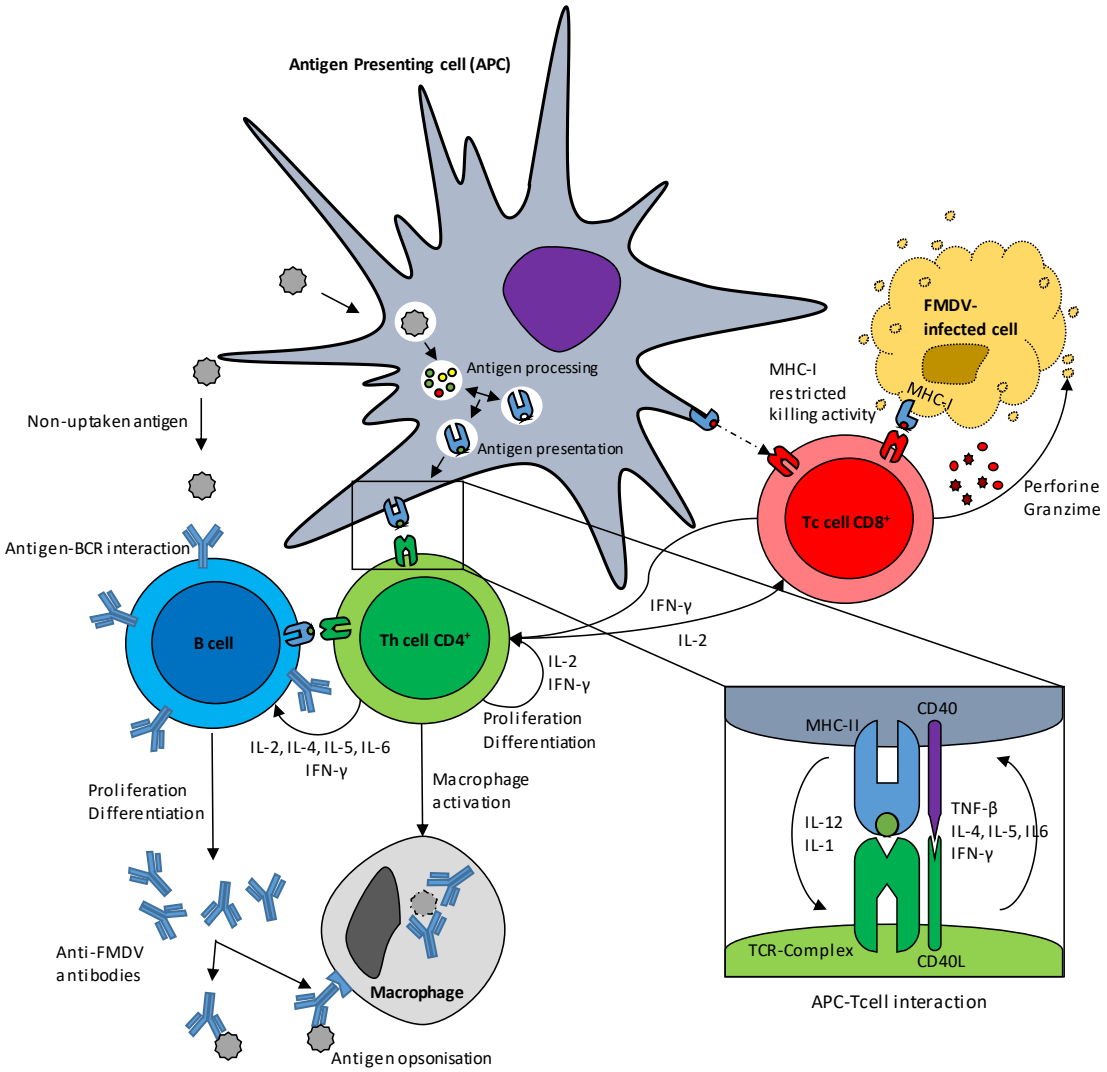


Figure 5: Overview of the adaptive immune response to FMDV. The interactions of antigen presenting cell (APC) such us a dendritic cell (DC) with T-helper lymphocytes (Th cell $CD4^+$) and Tcytotoxic (Tc cell $CD8^+$) in antigen presentation are indicated. The production of antibodies and pro-inflammatory cytokines (IFN-γ) are critical to clear the infection.

Protection against FMD is often, but not always, associated with the induction of high levels of circulating nAbs, albeit animals with low levels of nAbs may become protected (McCullough et al., 1992a). These observations are consistent with effector humoral immunity involving more than antibody and with the phagocytic system being involved in removing virus-antibody complexes and destroying the virus (McCullough and Sobrino, 2004). Indeed, phagocytosis of virus-antibody complexes, following viral opsonisation, has been proposed to mediate viral clearance *in vivo* (McCullough et al., 1992a; McCullough et al., 1992b).

In natural hosts, nAbs can be detected in serum as early as 3 to 4 days upon infection or vaccination, being IgMs those first identified, reach a maximum at days 10 to 14 post-infection, and then wane. IgGs are detected between 4 to 7 days post-infection and become the major nAbs after two weeks (Francis and Black, 1983). In both, infected and conventionally vaccinated natural FMDV hosts, a positive correlation between nAb levels and protection is noticed (Mulcahy et al., 1990). Early upon infection or vaccination, a detectable antibody response can be detected in the secretions of the upper respiratory and gastrointestinal tracts (Francis and Black, 1983). IgM is the major antibody subclass found in early secretions (Diaz-San Segundo et al., 2006), being posteriorly followed by IgA and IgG as the time post-immunization (pi) progresses (Salt, 1993).

Early evidence of the involvement of antibodies in protection against FMDV drove the attention to the humoral response elicited by the virus. Nevertheless, different findings in the past decades support that T-cell responses also contribute to the protective immunity to this virus, reviewed in (McCullough et al., 2017; Sobrino et al., 2001). In cattle and pigs, B-cell activation and antibody production are associated with a lymphoproliferative response mediated by T-cells (mainly $CD4^+$) that recognise a number of viral epitopes, located in both, capsid proteins (Blanco et al., 2000; Collen et al., 1991; Garcia-Valcarcel et al., 1996; Gerner et al., 2009; Liu et al., 2011; Saiz et al., 1992; van Lierop et al., 1994) and NSP (Blanco et al., 2001; Garcia-Briones et al., 2004; Gerner et al., 2006; Rodriguez et al., 1994). These T-cell responses mediated by CD4 cells are expected to be required for protective immunity against FMDV, by participating in the production of antiviral antibodies and by maintaining the appropriate microenvironment needed for a synergistic immune response. Indeed, the transient lymphopenia observed in pigs early after FMDV infection is consistent with the relevance of specific T-cells in the protective response in this species (Bautista et al., 2003; Diaz-San Segundo et al., 2006; Golde et al., 2008). FMDV-specific CD8 T-cell responses have also been found in conventionally vaccinated pigs and cattle (Guzman et al., 2010; Saiz et al., 1992). The experimental difficulties for the functional analysis of FMDV-specific effector CD8 T-cells has hampered the understanding of the role of this cell subset in protection (Childerstone et al., 1999; Rodriguez et al., 1996). Factors potentially affecting these assays are: the rapid cytopathic effect produced by FMDV in cultured cells, the rapid inhibition of MHC-I expression upon infection that leads to an impairment of the presentation of viral peptides to CD8 T-cells and facilitates viral escape (Sanz-Parra et al., 1998), and difficulties in depleting CD8 populations *in vivo* (Juleff et al., 2009; Naessens et al., 1998). So far, although efforts

have been invested, including bioinformatic predictions, to identify FMDV cytotoxic epitopes (Gao et al., 2006; Liao et al., 2013), only one such epitopes, corresponding to VP1 residues (71-79), has been reported to be recognized by cattle associated with a specific MHC-I haplotype (Guzman et al., 2010).

2.5 FMD vaccines: current perspectives

2.5.1 Conventional vaccines

Vaccination has been by far the most effective method to control FMD (Parida, 2009). The current type of vaccines approved by the OIE, consist on chemically inactivated whole viruses that are emulsified with different adjuvants [reviewed in (Smitsaart and Bergmann, 2017)]. These vaccines have demonstrated their success in eliciting protective immunity against the disease in endemic countries. Nevertheless, inactivated vaccines show several disadvantages which led to adopt a non-vaccination (*stamping-out*) policy in the EU and Western countries. In case of an outbreak in regions where vaccination is not implemented, massive culling of susceptible animals has been the main measure to control the spread of the disease, leading to large economic losses due to restriction of animal movement and food industry (Grubman and Baxt, 2004).

Only when cell culture systems became available and binary ethyleneimine (BEI) was reported to be an efficient and safe inactivating agent for RNA viruses (Bahnemann, 1972; Mowat and Chapman, 1962), the vaccine production was totally established; a fact that was not fully achieved until mid-twentieth century.

When adequately formulated FMD inactivated vaccines induce protective responses. Nevertheless, many efforts have been paid to palliate some of their drawbacks, such us: (i) the requirement of a constant cold-chain (4ºC) to preserve thermal stability of FMDV particles whose immunogenicity decreases significantly due to its disassociation at higher temperatures into pentamers (see below), (ii) BSL-3 facilities are needed for production of live viruses, and (iii) the problems associated with ensuring the DIVA (**d**ifferentiating **i**nfected from **v**accinated **a**nimals) condition of vaccines to allow serological distinction between infected and vaccinated animals. Furthermore, the high variability among FMDVs that is reflected in 7 serotypes and multiple antigenic variants (Grubman and Baxt, 2004; Sobrino and Domingo, 2001) add more complexity to the development of a unique protective vaccine (Taboga et al., 1997). So far, the epidemiology status of each FMD endemic region determines the vaccine formulation, which frequently includes more than one FMDV serotype depending on the country. On the other hand, FMD-free countries are concerned about the potential threat of disease introduction, which was

reflected in the creation early this century of Vaccines Banks in EU and North America where ready-to-use vaccines are stored. The emergency vaccines envisaged to be produced from Vaccine Banks differ from conventional ones in that they contain higher amounts of antigen that enable them to produce rapid protective responses even at 4 days post immunization, as well as to avoid viral shedding from oropharynx from infected animals (Doel and Pullen, 1990). On the contrary, these emergency vaccines are time-limited, as amount of antigen has to be replaced every 12-18 months and require the use of liquid nitrogen to store the antigens.

For these reasons, the search of alternative vaccines is a topic of intense research since decades, reviewed in (Blanco et al., 2017; de Los Santos et al., 2018). A summary of the vaccine strategies explored to date is presented in **Table 1**, and those considered more relevant are commented in more detail below.

Table 1: Classical and alternative experimental approaches for FMD vaccines

Type	Serotype	Description	Species[a]	Reference
Chemically-inactivated virus	All	BEI-inactivated virus emulsified with different adjuvants. Current available FMD vaccine. Vaccine strain selection depends on the epidemiological status of each country.	**FMD-susceptible animals**	(OIE, 2018)
Live attenuated vaccines	A	Virus lacking L^{pro} coding sequence (leaderless)	**Cattle/Swine**	(Chinsangaram et al., 1998; Mason et al., 1997)
		Mutations at the L^{pro} coding sequence (SAP mutations) afforded early protection (2 dpi)	**Swine**	(Segundo et al., 2012)
		Deoptimized virus by multiple synonymous mutation in the capsid coding region	**Mice**/Swine	(Diaz-San Segundo et al., 2015)
		RGD-deleted viruses	**Cattle**	(McKenna et al., 1995)
Live vectored vaccine	A	Recombinant replication-defective human adenovirus type 5 (Ad5) containing P1 and $3C^{pro}$ coding regions	**Swine/Cattle**	(Mayr et al., 1999; Moraes et al., 2002; Schutta et al., 2016; Sitt et al., 2019)
		Modified vaccinia virus Ankara (MVA) expressing P1 and a mutant version of $3C^{pro}$	**Cattle**	(Steigerwald et al., 2019)
	C	Recombinant vaccinia virus expressing P1	**Swine/ Mice**	(Berinstein et al., 2000; Sanz-Parra et al., 1999)
Empty Viral-like particles (VLPs)	A	Capsid proteins (empty capsids) expressed in *E.coli*	**Swine**	(Grubman et al., 1993)

Continuation

Type	Serotype	Description	Species[a]	Reference
	Asia1	Large scale production of VLPs from capsid coding regions expressed in E.coli	**Cattle**	(Xiao et al., 2016)
	O	VLPs and $3C^{pro}$ expressed in an self-replicative RNA from Semliki Forest Virus	**Cattle**	(Gullberg et al., 2016)
	A/O	Recombinant hepatitis B particles harboring B and T-cell FMDV epitopes	Mice	(Lei et al., 2019)
DNA vaccines	C/O	DNA vaccine targeting B and T-cell epitopes and improved versions coupling Bcl-xL protein	**Swine**	(Borrego et al., 2011; Ganges et al., 2011; Gulce Iz et al., 2013)
	Asia1	DNA vaccine expressing P1, 2A and 3C along bovine IL-18	**Cattle**	(Kotla et al., 2016)
	O	DNA vaccine containing P1, 2A, 3C and 3D coding regions	**Cattle**	(Fowler et al., 2012)
Peptide vaccines		VP1 linear peptide		(Bittle et al., 1982; DiMarchi et al., 1986)
	C	GH-loop linear peptide alone or linked to a T-cell epitope	**Cattle**	(Taboga et al., 1997)
	O	GH-loop linked to a heterologous T-cell epitope	**Swine**	(Wang et al., 2002)
	C/O	Dendrimer peptide displaying multiple B-cell epitopes linked to a Th-cell epitope	**Swine/Cattle**	(Blanco et al., 2016; Cañas-Arranz et al., 2020; Cubillos et al., 2008; Soria et al., 2017)(Cañas-Arranz, submitted)

[a]In bold, challenge experiments and protection

2.5.2 Live attenuated vaccines

As for many other viruses, non-naturally FMDV susceptible animals were initially used to select attenuated FMDV vaccines (Bachrach, 1968). This approach implies adaptation of FMDV isolates by serial passages in guinea pigs, mice, rabbits or embryonated eggs. However, it soon became evident the lack of guarantee of attenuation of theses adapted viruses in the different FMDV natural hosts (Bachrach, 1968; Brooksby, 1982; Nunez et al., 2007). Since then, a balance between attenuation and permissive replication to induce protective immunity in natural hosts has been pursued in the attempts to develop safe live attenuated vaccines. Targeting some of the FMDV virulence factors identified during the

past decades has been a major strategy to attempt development of live attenuated vaccines. This was the case of viruses lacking L^{pro} protein that exhibited features of attenuation and induced nAbs in cattle (Mason et al., 1997) and swine (Chinsangaram et al., 1998). Moreover, mutations in the coding region of L^{pro} render viruses that elicit very early protective immunity (Segundo et al., 2012). The immunogenic potential of FMDVs with mutations in the cell receptor binding motif, RGD at VP1 capsid protein has also been assessed. Viruses lacking this region were attenuated in cultured cells and in swine, and challenge experiments showed that were able to afford nAbs and complete protection in cattle (McKenna et al., 1995).

Recent studies reported that engineered viruses with multiple synonymous nucleotide substitutions in the coding region of structural proteins were viable in cell culture while being attenuated in swine (Diaz-San Segundo et al., 2015). This approach exploits the observation that the codon usage bias, a species-specific trait, can influence virus replication and virulence (Mueller et al., 2006). So far, this attenuation approach, called codon deoptimization, has not been tested in protection experiments in natural hosts. The $3D^{pol}$ protein has also been targeted for virus attenuation. Mutations altering the conformation of $3D^{pol}$ result in an impaired replicative ability in other picornaviruses (Campagnola et al., 2015). In this line, it has recently been shown that mutations affecting the fidelity of the RNA polymerase activity led to FMDV attenuation in animal models, becoming a potential model for vaccine development (Rai et al., 2017).

Overall, despite the unquestionable interest of developing attenuated FMD vaccines that may elicit a wide spectrum of immune responses similar to those observed in infected animals, the ample FMDV host-range and the high potential for variation of the virus make a careful study of the stability and pathogenicity of new recombinant vaccines necessary before they can be considered for field trials. In addition, the risk of reversion of virulence in natural hosts is still perceived as a concern being the major drawback for this type of vaccines.

2.5.3 Live-vectored vaccines

The use of viral vectors delivering FMDV antigens, has also been explored. Numerous reports and strategies have been documented, for example, the use of poxviruses such as vaccinia virus or fowlpox virus, expressing FMDV capsid coding regions (Sanz-Parra et al., 1999; Zheng et al., 2006). However, the limited protection they conferred in natural host has hampered their final consideration as a valuable vaccine.

The use of recombinant adenoviruses is a promising vaccine alternative. A recombinant human adenovirus type 5 (Ad5) expressing FMDV P1 and $3C^{pro}$ (Ad5-FMD) protected swine and cattle (Mayr et al., 1999; Moraes et al., 2002; Schutta et al., 2016; Sitt et al., 2019), in a process apparently mediated by the immune response elicited by the empty capsids produced (see next section). Moreover, very early protection could be achieved when it was administered along with type I IFN (Diaz-San Segundo et al., 2006; Moraes et al., 2003). Despite these encouraging results, high doses of recombinant adenovirus are needed to achieve protective responses, resulting in high cost manufacturing. Moreover, the efficiency of this type of vaccines may vary among serotypes, as a recombinant Ad5-FMD against serotype O virus fail to protect pigs upon homologous viral challenge (Caron et al., 2005). Thus, further studies with live-vectored vaccines seem necessary to confirm their value as future FMDV vaccines (de Los Santos et al., 2018).

2.5.4 FMDV-like empty particles

Whole empty viral capsids, also termed viral-like particles (VLPs), mimic the intact FMDV particle lacking infectious RNA. Its use as vaccine has been one of the main areas of research in the past decades (Rodriguez and Grubman, 2009). The advent of the cDNA technology and the molecular cloning made possible the expression of capsid proteins in bacteria and baculoviruses leading to recovery of empty capsids that were immunogenic in natural hosts (Grubman et al., 1993; Roosien et al., 1990). Recently, it has been reported that these particles can induce protective immune responses and are considered as DIVA vaccines, since no NSP proteins can contaminate their preparations. Nevertheless, the yield of empty capsids production is very low, limiting the feasibility viability of this approach for vaccine development (Xiao et al., 2016). Furthermore, the labile thermal nature of FMDV capsid leading to its dissociation into pentamers also contributed to this limitation. As commented above, pentamers are poor immunogens due, among other reasons, to its trend to reassociate in an inside-out conformation *in vitro*, most likely hampering the exposure of the antigenic sites *in vivo* (Malik et al., 2017). As described in previous sections (see 2.2), the efficient expression of FMDV empty viral capsids requires the adequate processing of the P1 polyprotein by $3C^{pro}$. Although the cytotoxicity of $3C^{pro}$ in culture cells has limited this approach for years (Porta et al., 2013), down regulation of $3C^{pro}$ in the context of a bicistronic system can lead to a more stable and efficient production of empty particles (Gullberg et al., 2013), opening an interesting alternative for the improvement of vaccines based on these particles. Since one of the

major drawbacks for the use empty capsids as vaccines is their thermal stability (empty capsids are more sensitive to heat than virions), attempts are being focused on understanding the molecular basis of this lability and to modify the viral capsids to generate more stable particles, reviewed in (Mateu, 2017). Thus, mutations that introduce disulfide bonds between pentamers, led to more thermostable empty capsids that elicited nAbs in similar levels to that of parental capsids and afforded partial protection against virus challenge in cattle (Porta et al., 2013). Likewise, introduction of mutations in capsid protein VP2, predicted to establish non-covalent interactions that increase pentamer-pentamer stability in types O and SAT2 FMDVs, rendered more stable capsids. After short-term storage at 4ºC, these engineered capsids elicited in calves similar neutralizing titers than parental capsids. Remarkably, after long-term storage (4-6 months) mutant SAT2-type capsids were able to produce consistent protection in guinea pigs (Kotecha et al., 2015).

2.5.5 DNA vaccines

The use of recombinant DNA vectors expressing FMDV capsid proteins is a vaccine approach that has been also investigated (Beard et al., 1999; Cedillo-Barron et al., 2001). Besides the benefits concerning biosafety and storage, DNA vaccines also allow incorporation of several protective antigens, co-expression of those determinants that drive the antigen directly to APCs such as the incorporation of single chain variable fragments (scFv), as well inclusion of marker genes to generate DIVA vaccines. This strategy was followed by Borrego and co-workers that engineered a construction encoding the B-cell epitope (VP1 G-H loop) and a combination of two T–cell epitopes –[3A (21-25)] and [VP4 (20-34)]– fused to scFv targeting MHC-II molecules. Immunization with this DNA vaccine resulted in a delay in the disease onset and a reduced severity of clinical signs after FMDV challenge. Remarkably, an exacerbation of clinical signs was noticed in pigs immunized with a similar construction lacking the scFv, supporting the relevance of antigen targeting for eliciting effective DNA protective immunity (Borrego et al., 2011; Ganges et al., 2011; Gulce Iz et al., 2013).

2.5.6 Peptide vaccines

Targeting VP1 for the induction of neutralizing anti-FMDV antibodies was among the first attempts to produce peptide-based subunit vaccines as an alternative for conventional ones. Among the advantages of these peptide subunit vaccines are: (i) safety; as a non-

infectious material is required, and no reversion to virulence is possible, (ii) DIVA condition, (iii) easy to handle and store (no cold chain is required), (iv) chemical stability and (v) large scale production is affordable. The first attempts to produce synthetic whole VP1-based vaccines were reported in the early 80s (Kleid et al., 1981), however later reports evidenced the low immunogenicity of VP1, probably due to non-native folding in solution, discouraging the initial expectations.

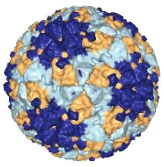
Bittle and coworkers, showed that a peptide corresponding to the G-H loop in VP1 (see 2.3.2), induced nAbs in mice and protection in guinea pigs (Bittle et al., 1982). Later, it was shown that this peptide linearly juxtaposed to the C-terminal of VP1, previously reported to induce nAbs (Strohmaier et al., 1982), was able to protect cattle from virus challenge (DiMarchi et al., 1986). An important advantage of this B-cell epitope in the VP1 GH-loop is that is structurally continuous and easy to mimic as a peptide (Cubillos et al., 2008; Wang et al., 2002). In contrast, the discontinuous or conformational-dependent structure of other neutralizing FMDV B-cell epitopes (Liu et al., 2017; Mateu et al., 1998) still hamper their mimicry using synthetic peptides and, therefore, their use for vaccine purposes (Villen et al., 2002; Villen et al., 2006). Despite the vaccine potential of peptides, the main problem faced during decades was their weaker immunogenicity when compared with conventional, inactivated vaccines (Doel et al., 1988). Moreover, the MHC restriction phenomenon can limit the recognition of certain T-cell epitopes in different individuals. For this reason, inclusion of T-cell epitopes recognized by different MHC molecules (promiscuous) capable of evoking adequate T-cell responses, are required to optimize the production of FMDV-nAbs. As discussed in previous sections (see 2.4), there are reports of T-cell epitopes recognized by lymphocytes from different FMDV hosts (Sobrino et al., 1999). Indeed, inclusion of a T-cell epitope identified in cattle [VP1 (21-40)] (Collen et al., 1991) in constructions linearly juxtaposing peptides corresponding to the G-H loop and the site C in VP1 resulted in an improvement in the protection they afforded (Taboga et al., 1997). In this large scale vaccination trial, escape mutants were isolated from unprotected animals, highlighting the potential for virus variation in response to vaccines including a limited number of epitopes, such as peptide vaccines.

Since classical linear peptides barely achieved levels of protection in livestock as those required for their use as commercial vaccines, multimerization strategies has been developed to overcome this low-immunogenicity. Thus, multiple copies in tandem of the VP1 G-H loop produced in in bacteria from different FMDV serotypes linked to a T-cell

epitope, have been reported to elicit in mice anti-FMDV responses similar to those of commercial FMD vaccine (Lee et al., 2017). A more complex multimerization approach is the so-called multiple antigenic peptides (MAPs), in which, using a single scaffold molecule, the B-cell epitope is branched from a core of lysines resulting in a dendrimer peptide (Cubillos et al., 2008; Tam, 1988). Interestingly, one of this dendrimer peptides displaying four copies of the G-H loop from a type C FMDV linked to a heterotypic and highly conserved T-cell epitope from FMDV 3A protein [3A (21-35)], was able to protect pigs against homologous FMDV challenge (Cubillos et al., 2008) upon administration of two doses of 2 mg of peptide each. Remarkably, downsized versions bearing two copies of the B-cell epitope afforded full protection in swine against an epidemiologically relevant type O FMDV (Blanco et al., 2016), using the same immunization schedule. Moreover, these dendrimer constructions have been recently shown to protect cattle harboring a previously reported bovine T-cell epitope (VP1 [21-40]) (Collen et al., 1991) albeit, in this case, after three immunizations. In this study, although low IgG2 titers were obtained, high IgG1 anti-FMDV levels were found, consistent with anti-FMDV neutralization and FMDV-opsonization in protective responses in cattle (Soria et al., 2017).

These encouraging results are on the basis of the objectives of this Doctoral Thesis that aimed at providing further support for the development of alternative vaccines based on these dendrimer constructions, by addressing different aspects relevant for vaccine feasibility using the pig as a model.

For a rational peptide vaccine design, the inclusion of adequate T-cell epitopes and the incorporation of the B-cell antigenic site in a functional conformation capable to be efficiently recognized by B-cells, are critical issues for optimizing the protective response elicited and reducing the amount of antigen and/or the number of vaccinations required to confer protection. As commented above, the continuous FMDV VP1 G-H loop has been proven to play an important role in protective immunity. Moreover, several T-helper epitopes haven been described in swine and cattle and, although it is clear that much remains to be learnt, such as the role of CD8 T-cells, the development of synthetic dendrimer vaccines offers considerable interest. The dendrimeric approach also offers the possibility of including different B- and T- cell epitopes either in the same molecule or in mixtures of dendrimers. Moreover, these heterologous platforms can prevent potential selection of antigenic variants in non-protected vaccinated animals.



OBJECTIVES

3. Objectives

Previous results from our group had shown the capacity of dendrimer peptides encompassing the B-cell peptide VP1 and the T-cell peptide 3A to confer protection against FMDV challenge. Nevertheless, no information was available on the role of different T-cells subsets on the protective response elicited, their duration and dose-dependence, as well as the potential of other FMDV T-cell peptides to modulate these responses. These points are relevant to understand the immune mechanisms driving dendrimer peptide immunogenicity and consequently important aspects for vaccine design. Therefore, the objectives of this Doctoral Thesis were:

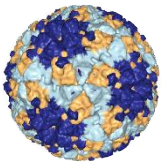
1. To study the immune response elicited in mice by different dendrimer peptides and selection of those more immunogenic for further characterization.

2. To study the effect of peptide amount and the number of doses on the protective responses elicited by B_2T-3A and its dimeric version B_2T-TB_2 and their duration, using an important natural FMDV host: the pig.

3. To evaluate the clinical protection against FMDV challenge conferred by two amounts and a single dose of B_2T-3A in pig.

4. To characterize the antibody response and the T-cell populations stimulated upon immunization of pigs with B_2T-3A and other dendrimer peptides incorporating different T-cell epitopes.

5. To optimize FMDV dendrimer peptides avoiding non-professional immune cells and potential induction of broad anti-FMDV immunity.



MATERIALS AND METHODS

4. Materials and Methods

4.1 Cell cultures

The following cell lines were employed:

- BHK-21: baby hamster kidney cells (*Mesocricetus auratus*) (Stoker and Macpherson, 1964).
- IBRS-2: porcine kidney cells (*Sus scrofa*) (De Castro, 1964).

Cell lines were maintained in Dulbeco's modified Eagle medium (DMEM) (Gibco), supplemented with 100 U/ml of penicillin (Sigma), 2 mM L-glutamine (Gibco) (complete DMEM) and 10% fetal bovine serum (SFB), at 37º C, 7% CO_2 and 98% humidity.

Cells were stored in liquid nitrogen following a slow freezing process with 90% FBS and 10% DMSO (Sigma). To thaw, cells were introduced in a 37ºC bath, centrifuged 250 x g for 5 min, resuspended and distributed in cell cultured plates in complete DMEM 10% FBS. Confluent monolayers were removed using 0.05 mg/ml of trypsin (Difco) and 0.016% EDTA (Merck) and cells were seeded in new plates at the required dilution (passage).

4.2 Viruses

The following FMDV strains were used in this study:

- O/UKG/11/2001: FMDV of serotype O from Middle East-South Asia topotype and OPanAsia lineage. The virus, which was recovered from two passages in IBRS-2 cells, was kindly provided by The Pirbright Institute (Surrey, UK) (Cottam et al., 2006) (GenBank: DQ404180).
- O_1Campos: FMDV of serotype O from Europe-South America topotype that was recovered upon three amplifications in IBRS-2 cells (GeneBank AJ320488). It was kindly provided by OPS-PANAFTOSA (Rio de Janeiro, Brazil) (Pereda et al., 2002).
- O_1BFS 1860/UK/67: FMDV of serotype O from Europe-South America topotype, which was recovered upon IDEM amplification in IBRS-2 cells four times (GeneBank AY593815) (Carrillo et al., 2005).
- O/SKR/1/2002: FMDV of serotype O from Middle East-South Asia, which was amplified in IBRS-2 cells five times (GeneBank DQ164972). It was kindly provided by The Pirbright Institute (Surrey, UK) (Knowles et al., 2005).

- O/Manisa/TUR/69: FMDV of serotype O from Middle East-South Asia topotype, which was amplified in IBRS-2 cells three times (GeneBank AY593823) (Carrillo et al., 2005).
- C-S8c1: FMDV of serotype C derived by three plaque purification from the field isolate C Santa Pau Sp/70 (Santa Pau, Girona 1970). (GeneBank AJ133357) (Sobrino et al., 1983).

4.3 Amplification of FMDV in cultured cells

The viruses detailed in 4.2 were amplified by infecting monolayers of BHK-21 cells (for C-S8c1) or IBRS-2 cells (for serotype O FMDVs) with a high multiplicity of infection [MOI; plaque forming units (PFU)/cell]. After 1 h adsorption at 37ºC, cells were washed with complete DMEM and fresh DMEM supplemented with 5% FBS for BHK-21 and 10% for IBRS-2 was added. When cytopathic effect was observed, whole cultures containing supernatants of infection and cell debris were collected and stored a -80ºC. Then, samples were subjected to three cycles of thawing-freezing to increase intracellular virus recovery and centrifuged at 250 x g for 10 min at 4ºC. The supernatant was collected, divided into aliquots and stored at -80ºC.

4.4 FMDV titration by plaque assay

Viral titers were determined by infecting BHK-21 cells (for C-S8c1) or IBRS-2 cells (for serotype O FMDVs) in multiwell 6 plates. For that, serial dilutions of the viral stock were used to infect duplicates of near-confluent monolayers as indicated in 4.3. After 1 h infection, the inoculum was removed and semisolid medium was added (0.5% agar, 1-2% FBS depending on the monolayer confluence, and 0.045 mg/ml DEAE-dextran in DMEM). After 24 h for C-S8c1 or 48 h for serotype O viruses, cells were fixed in 2% violet crystal and 2% formaldehyde. The plates were washed to remove the remaining violet crystal, allowed to dry at room temperature (RT) and the PFUs visualized were counted.

4.5 Peptides

The peptides were synthesized by Dr. D. Andreu's group (Universitat Pompeu Fabra). Two types of peptides were synthesized: i) linear peptides corresponding each to the B and T-cell epitopes used in this thesis that were used for *in vitro* stimulation assays (**Table 2**), and ii) dendrimers, which were used as immunogens as well as for *in vitro* stimulation

(**Table 3**). The general formula of the dendrimer peptide was B_nT where "B" is the B-cell epitope, "n" indicates the number of B copies and "T" the heterotypic and highly conserved porcine T-cell epitope. For detailed information regarding peptide synthesis, see Supplemental Information 8.1. The following dendrimer peptides were synthesized:

- B_2T-3A: containing two copies of the B-cell epitope from FMDV O/UKG/11/2001 [VP1 (140-158)] or C-S8c1 [VP1 (136-154)] (as indicated) (see 4.2), conjugated to a heterotypic and immunodominant porcine T helper epitope identified in FMDV NSP 3A [3A (21-35)] by a maleimide-thiol bond through the Michael-type thiol-ene reaction (Blanco et al., 2001).

Likewise, the following B_2T-3A related dendrimers were employed:

- B_2T-3D: incorporating, as T-cell epitope, that identified in FMDV NSP 3D, [3D (56-70)] (Garcia-Briones et al., 2004).

- B_2T-3C: incorporating, as T-cell epitope, that identified in FMDV NSP 3C [3C (166-180)] (Blanco et al., 2001).

- B_2T-3A (Br): equivalent to B_2T-3A, in which the B-cell epitope was linked to the T-cell by a maleimide group (Ramesh et al., 2015).

- B_2T-TB_2: containing two B_2T-3A molecules covalently joined tail-to-tail by copper(I)-catalyzed azide-alkyne 1,3-cycloaddition (Forner MF, Thesis 2020).

- B_4T: encompassing four copies of the B-cell epitope bound to the T-cell epitope 3A (21-35) (Cubillos et al., 2008).

- B_2T-3A3D: containing the two T- cell epitopes T-3A and T-3D linked in tandem.

- B_2T-3D3A: enclosing the two T-cell epitopes T-3D and T-3A linked in tandem.

- B_2: The two B-cell epitopes were attached to a bismaeimide-derivatized lys tripeptide core, lacking any T-cell epitope.

- B_2(scr)-T(scr): B_2T-3A dendrimer in which both, the B-cell and the T-cell epitope T-3A sequences were randomly scrambled.

- B_2T (R141H) and B_2T (R141K): B_2T-3A dendrimers whose B-cell epitope sequences corresponded to that of VP1 C-S8c1 isolate and contained the single amino acid substitutions VP1 R141H or R141K, respectively.

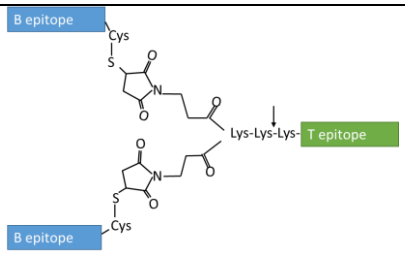
All the lyophilized peptides were dissolved in MilliQ water to a final concentration of 10 mg/ml and stored at -80ºC.

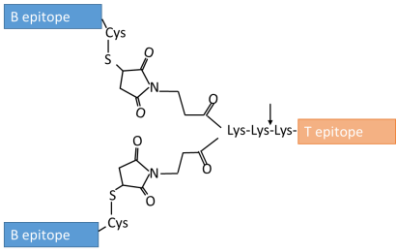
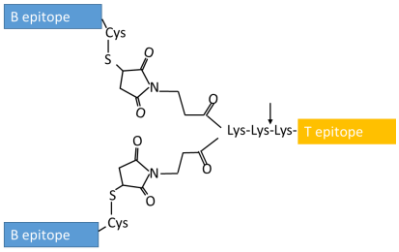
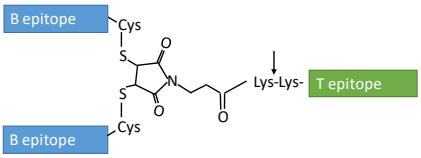
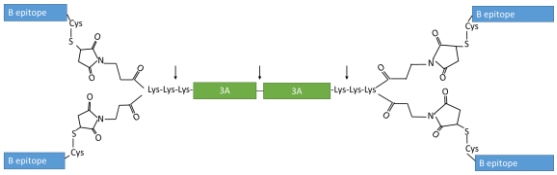
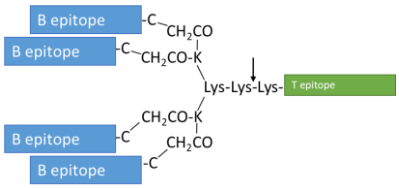
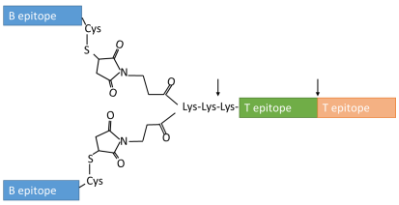
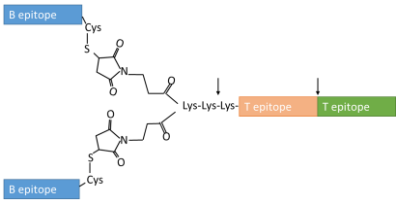
Table 2. Linear peptides corresponding to FMDV epitopes used in this study

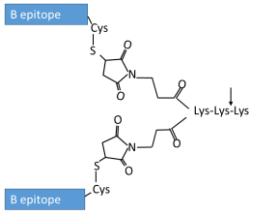
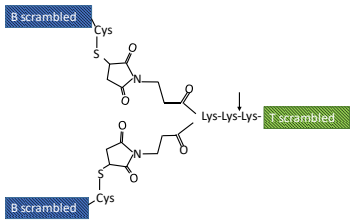
Peptide	Protein[a]	Amino acid sequence[b]	Residues	Reference
B (O/UKG/11/2001)	VP1 (2079)	PVTNV**RGD**LQVLAQKAART	140-158	(Cottam et al., 2006)
B (C-S8c1)	VP1 (2121)	YTASA**RGD**LAHLTTTHARH	136-154	(Toja et al., 1999)
T	3A (1693)	AAIEFFEGMVHDSIK	21-35	(Blanco et al., 2001)
	3C (1477)	AVLAKDGADTFIVGT	166-180	(Blanco et al., 2001)
	3D (1721)	IFSKHRGDTKMSAED	56-70	(Garcia-Briones et al., 2004)

[a] FMDV protein encompassing the peptide. In brackets the molecular weight in daltons (Da).
[b] From N-terminus to C-terminus. The RGD motif at the VP1 G-H loop is shown in bold.

Table 3. Dendrimeric peptides used as immunogens

Peptide[a]	Residues in FMDV proteins	Structural scheme[c]
B_2T-3A (6742.8)	VP1[b] (140-158) 3A[c] (21-35)	

B_2T-3D (6770.8)	VP1 (140-158) 3D (56-70)	
B_2T-3C (6526.6)	VP1 (140-158) 3C (166-180)	
B_2T-3A (Br) (6587.7)	VP1 (140-158) 3A (21-35)	
B_2T-TB_2 (14247.6)	[VP1 (140-158) 3A (21-35)] - [3A (35-21) VP1 (158-140)]	
B_4T (11205.1)	VP1 (140-158) 3A (21-35)	
B_2T-3A3D (8703.6)	VP1 (140-158) 3A (21-35) 3D (56-70)	
B_2T-3D3A (8703.6)	VP1 (140-158) 3D (56-70) 3A (21-35)	

B_2 (5066.9)	VP1 (140-158)	
B_2scr-Tscr[e] (6742.8)	VP1 (140-158) scrambled 3A (21-35) scrambled	

[a] Peptides are designated as in section 4.5. In brackets the molecular weight in daltons (Da).
[b] Sequence in VP1 corresponds to the B-cell site at the G-H loop of this protein from FMDV isolate O/UKG/11/2001 (see **Table 2**). For type C dendrimer this sequence was replaced by that of C-S8c1 isolate spanning residues 136-154 of VP1.
[c] The protein residues spanning the different T-cell epitopes are indicated.
[d] The B-cell epitope is indicated in blue and the T-cell epitope in different colors depending on the protein it was identified: 3A (green), 3D (pink) and 3C (yellow). The arrows indicate a target for cathepsine D, a protease suggested to be involved during in vivo major histocompatibility complex (MHC) class II antigen processing (Borras-Cuesta et al., 1988).
[e] B_2T-3A-like dendrimer containing scrambled B- and T-cell epitopes (TGRTVQANVLQDLPAKRA and HMAFESFDGIVKIAE sequences, respectively).

4.6 Animal experiments

The experimental design and the housing of the animals were in compliance with the current National and European regulations in animal welfare. Accordingly, the experiments detailed below were conducted under the approval of CSIC and INIA Committees on Ethics of Animal Experiments and Biosafety, as well as of the National Committee on Ethics and Animal Welfare (PROEX 034/15 at CBMSO; PROEX 218/14, PROEX 47/13 and PROEX 214/15 at INIA).

- Mice: To evaluate the immunogenicity of dendrimer peptides, groups of 6-7 weeks-old female mice (n=5-8) strain Swiss ICR-1 CD1® (Envigo) were used. Animals were maintained in the animal facility at CBMSO supplied with food and water *ad libitum*.

- Pigs: To study the immunogenicity and protection against FMDV O/UKG/11/2001 afforded by dendrimer peptides (see 4.2), female 2 months-old Landrace X Large White pigs (Agropardal breeding) were selected. These animals were immunized with the dendrimer peptides and were housed in the animal facilities at the Departamento de Reproducción Animal, INIA. Experiments involving viral challenge of pigs were performed at CISA-INIA.

4.7 Emulsion preparation and vaccine formulation

- Small scale vaccine preparation for mice immunization: Peptides were thawed at RT and water-in-oil emulsified in the oil adjuvant Montanide ISA 50 V2 (Seppic. France). To this end, the desired amount of peptide was diluted in PBS (aqueous phase) and was incorporated drop-by-drop while vortexing over the adjuvant (oil phase). The proportion of aqueous and oil phases was 1:1. Next, this emulsion was further stirred in a vortex for 2 min at RT and finally stored at 4ºC until use.

To prepare a larger scale emulsion to be used as inoculum for pigs the following approach, similar to that use for conventional FMDV vaccines manufacturing, was followed.

- High scale vaccine preparation for pigs: In this case, the vaccine formulation consisted of 40% of peptide and 60% adjuvant, as recommended by the OIE for commercial FMDV vaccines (OIE, 2018). Briefly, the aqueous solution of peptide was pre-emulsified 2 min at RT using vortex as described in the previous paragraph. The mixture was subjected to homogenization at 21.500 rpm using an ULTRA TURRAX T18 basic (IKA) kindly provided by Dr. Susana Cadenas (CBMSO) for 15 s at RT. Finally, the emulsion was stored at 4ºC until use.

4.8 Inoculation of animals

4.8.1 Inoculation of animals with dendrimer peptides

- Mice were immunized by subcutaneous route injection with two doses of 100 µg/animal of emulsified peptide in a final volume of 100 µl, administrated in an interval of 21 days.

- Pigs were immunized by intramuscular route injection with one or two doses of 2 or 0.5 mg of emulsified peptide in a variable interval (depending on the experiment). The final volume of inoculum was 2 ml for each pig.

4.8.2 Challenge of pigs with FMDV

Pigs (see 4.6) were challenged by intradermal injection in the coronary band of the hoof with 1.6×10^4 PFU of FMDV O/UKG/11/2001 (see 4.2) prior anesthesia. After challenge, the emergence of vesicular lesions and other clinical signs were monitored daily for 10 days. In non-immunized animals, vesicular lesions appeared from day 3 post-challenge (pc).

A protection score based on the day of emergence and the number and severity of lesions was determined. Total protection was defined as complete absence of lesions except at the point of inoculation. Partial protection was considered when mild and delayed symptoms were observed.

The clinical signs were quantified using a score based on FMD typical symptoms in pigs (maximum value of 7) (Cubillos et al., 2012): i) 1 point/vesicle in foot (up to 4 points); 1 point/mouth, tongue or snout lesion; 1 point/>2 lesions of diameters ≥ 10 mm.

4.9 Purification of PBMCs

Blood from immunized or infected pigs (see 4.6.2) was collected in Vacuette K3EDTA tubes (Greiner bio-one). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in a density gradient using Histopaque-1077. Briefly, 8 ml blood was centrifuged 15 min at 800 x g at RT in Leucosep tubes containing 3 ml of Histopaque-1077, and the cellular white band was recovered. Then, erythrocytes were lysed by 10-fold sample dilution in 150 mM NH_4Cl, 10 mM $NaHCO_3$ and 1,5 mM HEPES for 5 min at RT (Chernyshev et al., 2008). PBMCs were washed three times in PBS-EDTA 0.05%, centrifuged 10 min at 250 x g and resuspended in Roswell Park Memorial Institute medium (RPMI 1640) supplemented as in 4.1 plus 50 µM β-mercaptoethanol (Fisher Scientific) and 20 mM HEPES (Sigma). This medium, termed complete RPMI (RPMIc), was further supplemented with 10% FBS, which had been previously inactivated at 56ºC for 30 min. The number of live PMBCs was calculated using a Neubauer chamber (Immune Systems) and cell staining with 0.4% Trypan Blue (Sigma) in PBS. In general, fresh cells were used in the experiments and those remaining were cryo-preserved in 90% FBS and 10% DMSO in liquid nitrogen as in 4.1. The minimum amount of cells used for freezing was 2×10^7 / vial.

4.10 Determination of FMDV-specific IgG antibodies

Total anti-FMDV antibodies were determined by means of a sandwich ELISA (Blanco et al., 2013). Briefly, 96-well plates (High Binding, Nunc) were coated by overnight incubation at 4ºC with 1 µg peptide B diluted in coating buffer (150 mM Na_2CO_3, 30 mM $NaHCO_3$; pH 9.6). Plates were washed with PBS-Tween and blocked with 5% skimmed milk for 1 h at 37ºC. After washing the plates three times with PBS-Tween, duplicates of two-fold serial dilution of serum samples in 3% skimmed milk were incubated 1 h at 37ºC. The inoculum was removed and the wells were washed again three times as above

and incubated 1 h at 37ºC with the secondary antibody, an anti-mouse IgG (Invitrogen) labeled with peroxidase (for mice) or recombinant protein A labeled with peroxidase (Life Technologies) (for pigs), in 3% skimmed milk. The plates were washed three times with PBS-Tween, incubated with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Life Technologies) and the reaction was stopped after 15 min using the same volume of H_2SO_4 3N. The absorbance was measured at 450 nm and titers were expressed as the reciprocal of the last dilution giving the absorbance recorded in the control wells (serum at day 0) plus 2 SD.

4.11 Detection of nAbs against FMDV

The titers of nAbs in sera were determined using a standard micro-neutralization test performed in 96-well plates. To this end, triplicates of serial two-fold dilutions of each serum were incubated 1 h at 37ºC with 100 $TCID_{50}$ (50% Tissue Culture Infective Dose) of the corresponding FMDV. Remaining antiviral activity was determined in 96-well plates containing fresh monolayers of BHK-21 or IBRS-2 cells. End-point titers were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of the corresponding FMDV in 50% of the wells (OIE, 2018).

The antigenic relationship of viruses was calculated by the ratio r_1 = nAb titre against the heterologous virus/nAb titre against homologous virus, as reported (Bari et al., 2014).

4.12 Detection of isotype-specific anti-FMDV antibodies (IgG1 and IgG2) by ELISA

Specific anti-FMDV IgG1 and IgG2 antibodies were measured in pig sera samples using a modification of the above ELISA and as reported (Salt et al., 1996). Briefly, duplicates of 3-fold serum dilutions were prepared in 50 µl, starting at 1/50, as described in 4.11. Isotype-specific anti-IgG1 and anti-IgG2 antibodies (BioRad) were incubated 1/2000 in 3% skimmed milk for 1 h at 37ºC. The plates were washed three times with PBS-Tween and incubated with HRP-labeled anti-mouse (Invitrogen) for 1 h at 37ºC. After 4 washes with PBS-Tween, TMB substrate was added and the reaction was revealed as in 4.10.

4.13 ELISPOT IFN-γ

Quantification of IFN-γ secreting cells from immunized and control animals was performed using an ELISPOT assay. Briefly, purified PBMCs (see 4.9) were *in vitro* stimulated with either the corresponding dendrimer or peptides T or B. First, 96-well plates (High Protein Binding, Immobilon-P Membrane, Millipore) were coated with anti-

pig IFN-γ (5 µg/ml, BD Bioscience) overnight at 4ºC. Next, the plates were blocked with RPMIc 10% FBS for 2 h at RT. Then, cells were seeded in a volume of 100 µl at a final concentration of $2.5x10^6$ cells/ml and incubated with 50 µg/ml of each peptide. Phytohemaglutinine-M (PHA-M) from *Phaseolus vulgaris* (10 µg/ml, Sigma) and RPMI alone were used as a positive and negative controls of stimulation, respectively. The plates were incubated for 48 h at 37ºC, washed with milliQ water and PBS-Tween three times, and incubated with anti-pig IFN-γ biotin-labeled (2 µg/ml, BD Biosciences) for 2 h at RT with slight rocking. After three washes with PBS-Tween, the wells were incubated with a 1/100 dilution of streptavidine-peroxidase (BD Biosciences) for 1 h at RT. After a final wash with PBS-Tween (4 times) the reaction was revealed using the substrate set AEC (BD) following the manufacturer's instructions. Finally, the reaction was stopped by washing the plates with milliQ water and the spots developed were quantified using an ELISPOT reader (CTL, Germany) and the software ImageJ 1.51j8 (http://imagej.nih.gov/ij). Data was presented as spots forming cells per 10^6 cells. Spots from medium-stimulated cells were considered as background and subtracted.

4.14 Intracellular cytokine staining (ICS)

The antibodies used in the flow cytometry experiments are shown in **Table 4**. To detect the phenotype of cytokine-producing cells, intracellular staining of purified PBMCs (see 4.9) was performed using 5 x 10^6 PBMCs from each pig that were incubated or not for 18 h at 37ºC with peptide at a final concentration of 25 µg/ml. Brefeldine A (5 µg/ml), a drug that blocks the exocytic pathway, was added 10 h before collecting the cells. RPMI alone was used as a negative control (non-stimulated) and PHA-M (25 µg/ml) as positive control of the assay in a non-immunized animal in each experiment. Following three washes with PBS-EDTA supplemented with 5 % swine serum, cells were surface stained by incubation with selected mAbs for 30 min at 4ºC in PBS-EDTA supplemented with 5% swine serum. Cells were washed with PBS-EDTA three times and incubated with the corresponding secondary labeled antibodies for 30 min at 4ºC. The cells were washed three times with PBS-EDTA, fixed and permeabilized for 20 min at 4ºC using Cytofix/Cytoperm buffer (BD). Then, samples were washed with PERM-WASH buffer (BD) and incubated with mAb for the detection of intracellular IFN-γ or TNF-α for 30 min at 4ºC. Finally, the cells were washed three times with PERM-WASH buffer and fixed using a solution containing 2% paraformaldehyde (Electron Microscopy Science). Cells were centrifuged 3 min at 250 x g, resuspended in a volume of 200 µl PBS-EDTA

5% FBS (see below 4.18) and were analyzed in a flow cytometer FACS CantoA (BD). The data were acquired using the software FlowJo (www.flowjo.com/).

4.14.1 Phenotyping of IFN-γ producing porcine T-cells

For surface staining PBMCs were incubated with undiluted anti-pig CD4 (74-12-4, IgG2b kindly provided by Dr. Javier Domínguez, INIA) and anti-pig CD8β (PG164A, IgG2a EuroVet Veterinaria) diluted 1/100. Anti-mouse Alexa647 for CD4 and anti-mouse Alexa488 for CD8-β were used as secondary antibodies. IFN-γ was detected using anti-pig IFN-γ- PE (BD).

4.14.2 Characterization of $CD4^+$ cell subsets involved in effector responses

PBMCs were incubated with anti-pig CD4 as mention above and undiluted anti-pig 2E3 (kindly provided by Dr. Javier Domínguez). Anti-mouse Alexa647 for CD4 and anti-mouse FITC for 2E3 were used as secondary antibodies. IFN-γ was detected using anti-pig IFN-γ- PE.

4.14.3 Detection of multifunctional IFN-γ and TNF-α producing cells

For surface staining PBMCs were incubated with undiluted anti-pig CD4. Anti-mouse Alexa488 was used as a secondary antibody. For intracellular cytokine staining anti-IFN-γ- PE and anti-TNF-α-APC were employed.

4.14.4 Enrichment of $CD4^+$ cell subsets using specific antibodies

To obtain enriched cellular fractions containing $CD4^+$ cells, a MACS (*magnetic activated cell sorting*) equipment in combination with an anti-CD4 antibody (kindly provided by Dr. J. Domínguez) and LD columns (Miltenyi Biotec GMbh) were used. Around 10^8 thawed PBMCs were used, of which 4 x 10^6 cells were reserved for ELISPOT of IFN-γ and the rest were magnetically sorted using an anti-CD4 antibody (clone 74-12-4) . The cells were incubated for 30 min at 4ºC with anti-CD4 diluted 1/1000 in MACS buffer (PBS-EDTA 2% FBS) in a proportion of 50 µl of antibody/10^6 cells. After two washes with MACS buffer, samples were incubated with an anti-mouse IgG coupled to magnetic microbeads (Miltenyi Biotec GMbh) in a ratio of 200 µl of microbeads/4 x 10^8 cells. After washing to remove excess of microbeads, the cells were resuspended in 2.5 ml of MACS buffer and were pass through a LD column that was previously equilibrated following the manufacturer instructions. The labeled fraction (positive fraction) and the non-labeled fraction (negative fraction) were eluted in 2 and 3 ml of MACS buffer, respectively. The

purity of each fraction was determined by flow cytometry. Finally, the cells were counted (as in 4.9) and prepared for/processed for their analysis by an ELISPOT of IFN-γ.

Table 4: Antibodies used in flow cytometry

Antigen	Clone	Isotype	Target	Fluorochrome[a]	Labeling strategy	Source of primary Ab
Single cytokine staining CD4$^+$, CD8β^+, IFN-γ						
CD4[b]	74-12-4	IgG2b	T-helper lymphocytes	Alexa647	Secondary antibody[c]	(Rodriguez-Carreno et al., 2002)
CD8β	PG164A	IgG2a	T-cytotoxic lymphocytes	Alexa488	Secondary antibody[d]	EuroVet Veterinaria
IFN-γ	P2G10	IgG1	Porcine IFN-γ	PE	Directly conjugated	BD Biosciences
Phenotyping of CD4$^+$ subsets						
CD4[b]	74-12-4	IgG2b	T-helper lymphocytes	Alexa647	Secondary antibody[c]	(Rodriguez-Carreno et al., 2002)
2E3[b]	2E3	IgM	Naive T-helper lymphocytes	FITC	Secondary antibody[e]	(Revilla et al., 2004)
IFN-γ	P2G10	IgG1	Porcine IFN-γ	PE	Directly conjugated	BD Biosciences
Double cytokine staining CD4$^+$, IFN-γ, TNF-α						
CD4[b]	74-12-4	IgG2b	T-helper lymphocytes	Alexa488	Secondary antibody[f]	(Rodriguez-Carreno et al., 2002)
IFN-γ	P2G10	IgG1	Porcine IFN-γ	PE	Directly conjugated	BD Biosciences
TNF-α	MAb11	IgG1	Human TNF-α	APC	Directly conjugated	BD Biosciences
Fractioning of PBMCs by MACS						
CD4[b]	74-12-4	IgG2b	T-helper lymphocytes	-	-	(Rodriguez-Carreno et al., 2002)
CD172a[b,h]	BLIH7	IgG1	Antigen-Presenting cells	Alexa647	Secondary antibody[g]	(Alvarez et al., 2000)

[a] Fluorescein (FITC), Phycoerythrin (PE), Allophycocyanin (APC).
[b] Kindly provided by Dr. Javier Domínguez (INIA).
[c] Goat anti-mouse IgG2b-Alexa647, Fisher Scientific.
[d] Goat anti-mouse IgG2a-Alexa488, Fisher Scientific.
[e] Goat anti-mouse IgM-FITC, Bionova.
[f] Goat anti-mouse IgG2b-Alexa488, Fisher Scientific.
[g] Goat anti-mouse IgG1-Alexa647, Fisher Scientific.
[h] Only for cell staining, not for MACS fractioning.

4.15 Amplification of viral RNA

4.15.1 Extraction of viral RNA

Viral RNA was extracted from 200 µl sera using TRI Reagent (Sigma) following the instructions of the manufacturer. The RNA obtained was eluted in 20 µl of diethyl pyrocarbonate (DEPC)-treated water and stored in -80ºC until its use.

4.15.2 Quantification of viral RNA molecules using real-time RT-PCR

Viral RNA quantification was performed by RT-qPCR (Reverse Transcriptase-quantitative Polymerase Chain Reaction). Single stranded cDNA was synthesized from 1 µl of total RNA, using the enzyme RT SuperScript (Invitrogen) and primer A (5´-CACACGGCGTTCACCCA(A/T)CGC-3´) (Saiz et al., 2003), following 30 min incubation at 55ºC. The cDNA synthesized was amplified by qPCR using "Light Cycler RNA Master SYBR Green I" (Roche) on a "LightCycler" (Roche) equipment. The primers A (above) and B (5´-GACAAAGGTTTTGTTCTTGGTC-3´) used in this reaction allowed amplification of a 290 bp conserved sequence from 3D protein (Saiz et al., 2003). The amplification program was initiated at 95ºC for 10 min followed by 45 cycles of 10 s 95ºC, 5 s at 60ºC and 10 s at 72ºC. After amplification, an additional thermal denaturizing cycle (temperature ranged between 65ºC and 95ºC in 0.1ºC/s increments) was performed to obtain the melting curves of the RT-qPCR products and verify amplification specificity. For each sample, reactions were run in triplicate.

All the samples were characterized by a corresponding cycle threshold (CT) value. The number of viral RNA copies (VRC) were interpolated from a standard curve performed with 10-fold serial dilutions of a cDNA from the infectious clone pMT28, which codifies for the genomic RNA of C-S8c1 FMDV (Garcia-Arriaza et al., 2004). The lower quantification limit was determined to be around 5 x 10^3 VRC/ml of serum.

4.16 Inhibition of FMDV attachment by dendrimer peptides

To assess the capability of peptides to block FMDV attachment to cultured cells a modification of the method described by Mateu et al. (Mateu et al., 1996) was followed. To this end, BHK-21 cells washed with PBS supplemented with Ca^{2+} and Mg^{2+} were incubated for 45 min at RT with B_2T dendrimer variants incorporating amino acid substitutions R141H or 141K at the RGD motif of the B-cell epitope (VP1 residues 136-154 from type C isolate C-S8c1). The canonical B_2T and B peptides, containing the intact

RGD motif, were used as positive controls and the irrelevant peptide T-3A was used as a negative control. Peptides were diluted in PBS containing Ca^{2+} and Mg^{2+} at a final concentration of 25, 2.5 or 0.25 µM. The cells were washed and infected with 100 PFU of CS8-c1 for 45 min at RT, washed with DMEM and agar-containing medium was added. The plaques formed were counted as in 4.4.

4.17 Statistical analyses

The values presented in the graphs corresponded to the mean ± the value of the standard deviation (SD). The data were analyzed using a t-test or analyses of variance (ANOVA), followed by Tukey's post-hoc for multiple comparisons. Statistical analyses were conducted using the software GraphPad 6.1. In the figures, differences were considered statistically significant when the *p* value were below 0.05 and are indicated as $*p < 0.05$, $**p < 0.01$ and $*** p < 0.001$.

4.18 Solutions and buffers

PBS: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl

PBS-EDTA: 0.2 M EDTA in PBS

PBS-Tween: 0.05% Tween20 in PBS

ELISA coating buffer: 15 mM Na_2CO_3, 30 mM $NaHCO_3$; pH 9.6

ELISA blocking buffer: 5 % skimmed milk in PBS-Tween

ELISA sera-incubated buffer: 3 % skimmed milk in PBS-tween

ELISPOT coating buffer: PBS

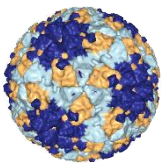
ELISPOT blocking buffer/peptide incubation: RPMI 10% FBS

Trypsin-EDTA: 0.5 mg/ml tripsine (Difco), 0.016 % EDTA, 0.0015 % red phenol, 1.12 mM glucose diluted in PBS

FACS antibody incubation: PBS-EDTA 5% swine serum

FACS fixation and permeabilization buffer: Cytofix and cytoperm: Commercial (BD)

Red Blood Cell lysis solution: 150 mM NH_4Cl; 10 mM $NaHCO_3$; 1.5 mM HEPES



RESULTS

5. Results

5.1 Analysis of the antibody response elicited by different FMDV dendrimers in the mouse model

The mouse has been used as a surrogate model to assess the immunogenicity of FMDV vaccines prior to their analyses in natural hosts (see **Table 1**). The mouse strain Swiss ICR (CD1®) offers the possibility of conducting immunogenic studies in outbred populations that mimic the heterogeneous genetic background of natural FMDV hosts. Previous results from our lab showed that the dendrimer peptide B_2T-3A, in which two copies of FMDV B-cell epitope [VP1 (140-158)] and one copy of the T-cell epitope from protein 3A [3A (21-35)] are held together through a maleimide link, was able to induce significant levels of nAb in outbred ICR mice. Since nAbs are considered as the main correlate of protection against FMDV (Blanco et al., 2013), this mouse strain was used to evaluate the immunogenicity of different dendrimers, including: i) those harboring T-cell epitopes other than that in 3A, present in the NSP 3D [3D (56-70)] (B_2T-3D) and 3C [3C (166-180)] (B_2T-3C) and previously identified in swine (Blanco et al., 2001; Garcia-Briones et al., 2004), ii) B_2T-3A tethered using maleimide bromide chemistry (B_2T-Br), iii) a tail-to-tail dimeric form of B_2T-3A termed B_2T-TB_2 and iv) a dendrimer B_4T harboring four copies of the B-cell epitope linked to 3A epitope, previously reported to induce nAbs in mice (Blanco et al., 2013) (see Materials and Methods 4.5). In this line, groups of five mice were immunized with the dendrimer constructions as shown in **Figure 6**. At day 21, the animals were boosted and at day 40 pi euthanized. Sera samples were collected at days 0, 21 and 40.

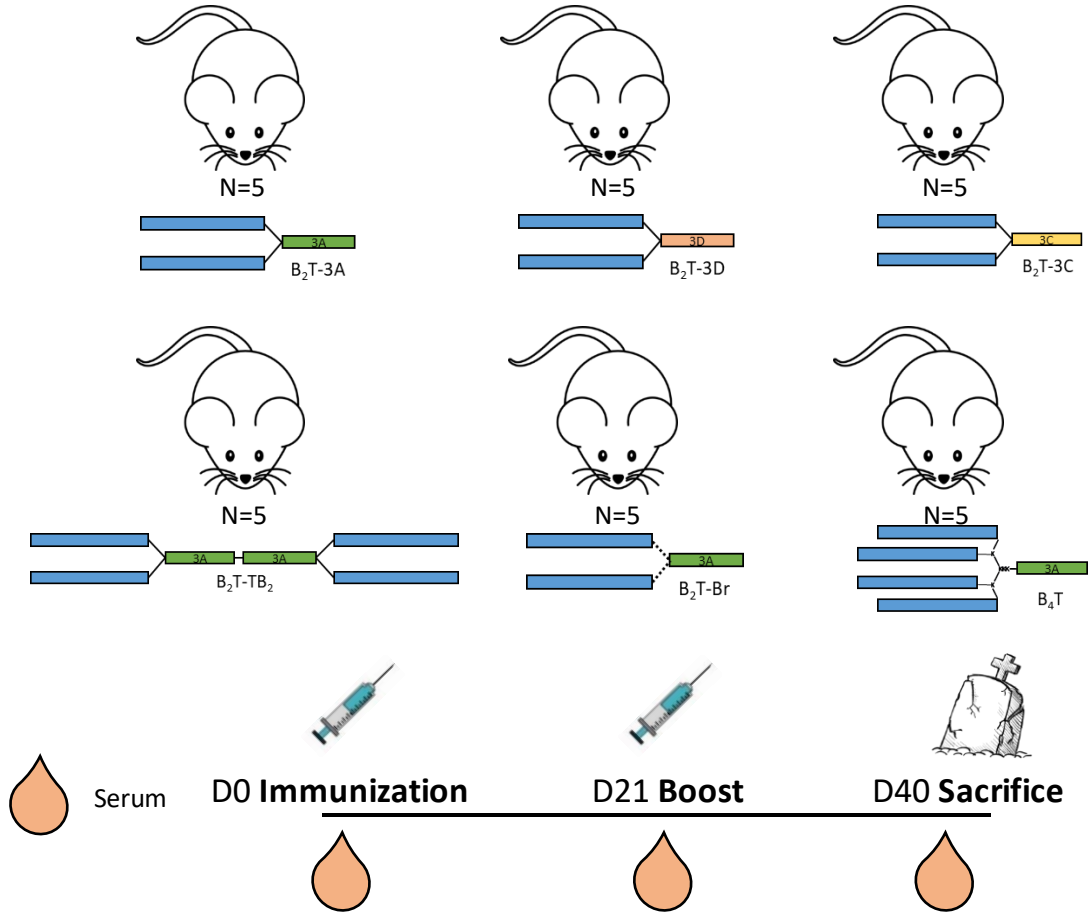


Figure 6: Experimental scheme followed to study the immunogenicity of different FMDV dendrimers in mice. Groups of five mice (n=5) were subcutaneously immunized with 100 µg of dendrimer at day 0. At day 21 the mice were boosted and sera samples were collected at day 0, 21 and 40.

Total IgG antibodies against B peptide were measured by ELISA after one dose (day 21 pi) and two doses of peptide (day 40 pi) (**Figure 7A and B**). After the first dose, the constructions B_2T-TB_2, B_2T-3A, B_4T and B_2T-BrM induced similar antibody titers in the immunized animals (3.5 ± 0.2; 3.4 ± 0.9; 3.3 ± 0.4 and 2.9 ± 0.9 $\log_{10}$, respectively), whereas B_2T-3C induced lower antibody titers (2.3 ± 0.5 $\log_{10}$). No antibodies were detected in any animal from the B_2T-3D group (**Figure 7A**).

After the peptide boost, antibody titers increased significantly in all groups. Among them, the construction B_2T-TB_2 induced the highest titers (5 ± 0.4 $\log_{10}$), followed by B_2T-3A (4.1 ± 1.2 $\log_{10}$), B_4T (3.9 ± 0.3 $\log_{10}$) and B_2T-Br (3.8 ± 1 $\log_{10}$). The titers from B_2T-3C group were lower (2.9 ± 0.8 $\log_{10}$) and only one animal from B_2T-3D group showed detectable antibodies (**Figure 7B**).

Next, neutralizing activity against homologous FMDV was analyzed in sera from immunized mice. At day 21 pi, nAbs were detected in animals from groups B_2T-TB_2 (1.4 ± 0.7 $\log_{10}$), B_2T-Br (1.2 ± 0.8 $\log_{10}$), B_2T-3A (1.1 ± 0.6 $\log_{10}$), B_4T (1 ± 0.4 $\log_{10}$) and

one animal from B_2T-3C (1.4 log_{10}). In contrast, none of the mice immunized with B_2T-3D displayed detectable nAbs (**Figure 7C**). After the boost, the titers increased in all groups. The construction that induced the highest levels was B_2T-TB_2 (2.6 ± 0.2 log_{10}) followed by B_2T-3A (2 ± 0.5 log_{10}), B_2T-Br (1.9 ± 0.9 log_{10}), B_4T (1.8 ± 1.1 log_{10}), B_2T-3C (1.4 ± 0.7 log_{10}) and B_2T-3D (< 1 log_{10}) (**Figure 7D**).

According to previous data (Blanco et al., 2013), the dendrimer peptide B_4T did not elicit nAb titers as high as those induced by B_2T-3A.

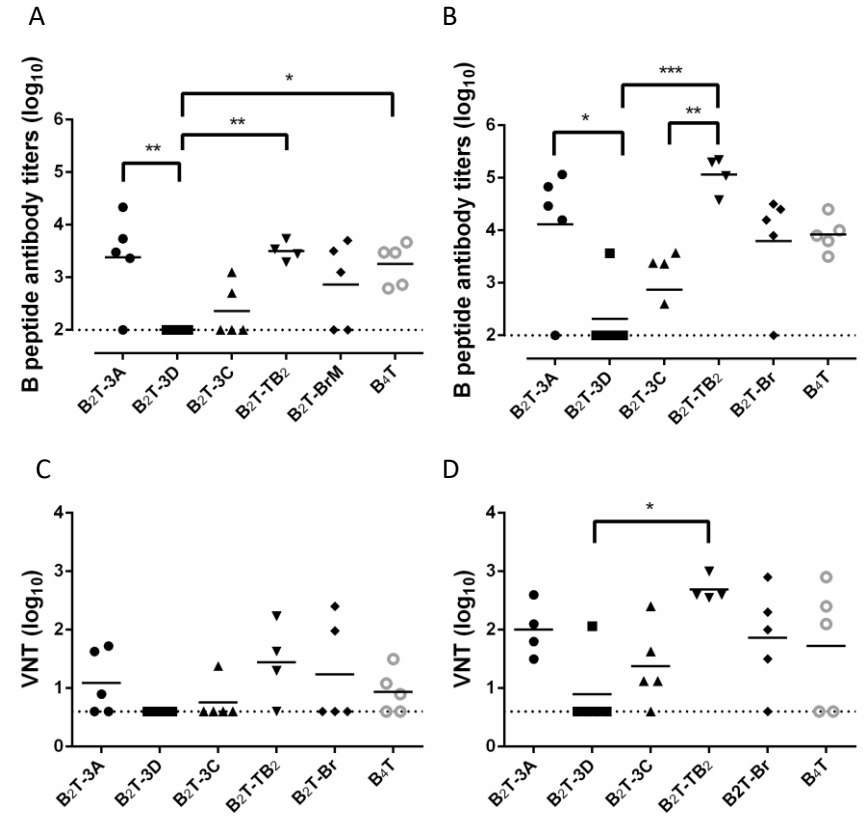


Figure 7: FMDV antibodies elicited in mice by different FMDV dendrimers. Total IgG antibodies against peptide B detected by ELISA in sera from immunized mice after the first (day 21 pi) (A) and the second peptide dose (day 40 pi) (B). Virus neutralization titers, expressed as the reciprocal log_{10} of the last serum dilution that neutralized 100 TCID50 of homologous FMDV, after the first (C) and second peptide dose (D). Each point represents the mean of a triplicate value of a single animal. Horizontal bars indicate the mean of each group. Statistically significant differences are indicated by asterisks (*) for $p < 0.05$, (**) for $p < 0.005$ and (***) for $p < 0.001$. A representative experiment out of five is presented.

5.2 Analysis of the long-term immune response induced by B_2T-3A and B_2T-TB_2 in pigs

5.2.1 Peptides B_2T-3A and B_2T-TB_2 elicit specific antibodies that can be detected five months upon peptide immunization

As commented previously (section 5.1) there is a correlation between the titers of nAb induced in mice and those elicited in pigs by peptide B_2T-3A (Blanco et al., 2013; Blanco et al., 2016). Thus, the encouraging results obtained with dendrimer B_2T-TB_2 in mice (see 5.1) prompted us to study the immune response elicited by this peptide in a natural FMDV host: the pig. Moreover, since the induction of long-term protective immunity is an essential requirement for efficient vaccines, we explored the durability of the immune response elicited by such dendrimer constructions. To that end, groups of four domestic pigs were immunized and boosted (day 39 pi) with 2 mg of B_2T-3A (pigs 76, 77, 78, 79), 2 mg of B_2T-TB_2 (pigs 81, 82, 84, 86) or 0.5 mg de B_2T-TB_2 (pigs 87, 88, 89, 90). Two non-immunized PBS-inoculated animals were used as controls (pigs 91, 92). Sera samples and PBMCs were collected at different days pi (**Figure 8**).

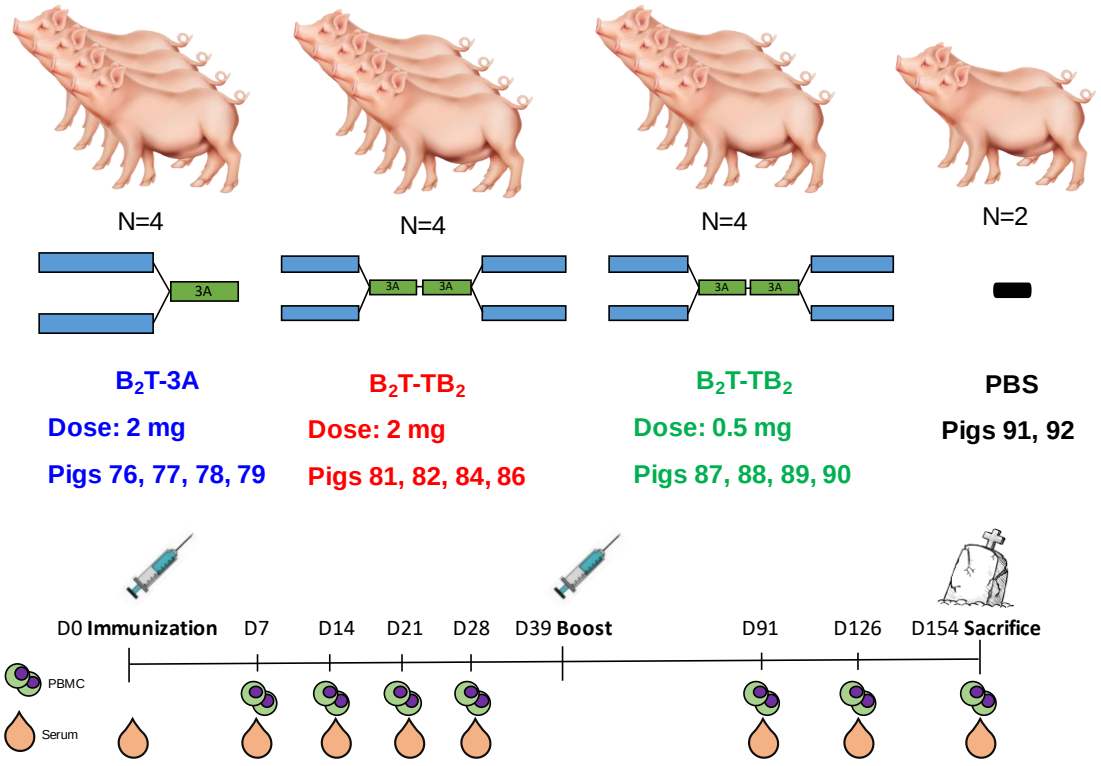


Figure 8: Experimental scheme followed to study the duration of the immunity elicited by B_2T-3A and B_2T-TB_2. Groups of four female 3 months-old domestic pigs (Landrace X White pigs) were immunized with B_2T-3A (2 mg), B_2T-TB_2 (2 mg) or B_2T-TB_2 (0.5 mg). Two non-immunized pigs were kept as controls. At day 39 pi animals were boosted and the time course of the immune response was analyzed until the end of the experiment at day 154 pi (5 months pi), when the animals were euthanized. Sera and PBMCs samples were collected at the indicated days.

The antibody titers to peptide B induced by B_2T-3A and by both doses, 2 and 0.5 mg, of B_2T-TB_2 were similar and no appreciable differences were observed among them. After immunization, antibody response reached a peak by day 28 pi (4 log_{10}). Remarkably, upon peptide boost, the titers were maintained in all cases up to day 154 (about 5 months pi). No specific antibodies were observed in non-immunized pigs (**Figure 9**).

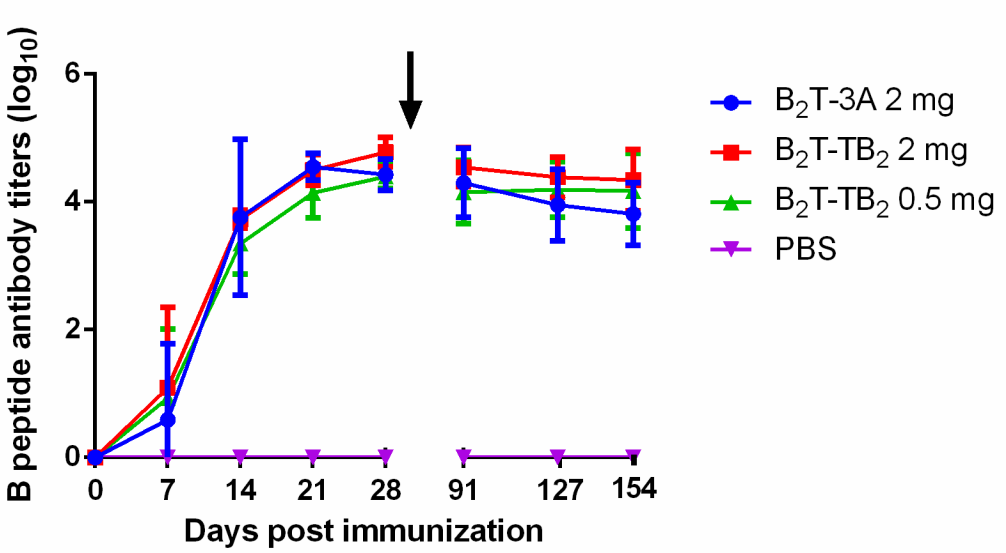


Figure 9: Time course of IgG antibodies to peptide B measured by ELISA in sera from B_2T-3A and B_2T-TB_2 (2 mg or 0.5 mg) immunized pigs. Each point depicts mean antibody titers (calculated as described in Material and Methods) ± 2SD for each group of immunized (n=4) or PBS-inoculated pigs (n=2). The arrow indicates the day of the boost.

5.2.2 Peptides B_2T-3A and B_2T-TB_2 elicit high and long-lasting levels of nAb

The presence of FMDV-specific nAbs against the homologous virus O/UK/11/2001 was analyzed in sera from pigs at different days pi. As shown in **Figure 10A**, no nAb were detected at day 7 pi in B_2T-3A immunized pigs. It was by day 14 when the significant titers were first observed (1.2 ± 0.4 log_{10}), increasing at day 21 pi (1.5 ± 0.5 log_{10}) and showing a slight decrease at day 28 pi (1.2 ± 0.4 log_{10}). After the boost, the nAb titers notably increased, reaching the highest values at day 91 pi (2.1 ± 0.4 log_{10}) and gradually decreasing by day 127 pi (1.7 ± 0.1 log_{10}). At the end of the experiment, day 154 pi, the titers were still detectable (1.6 ± 0.6 log_{10}), especially for pigs 76 and 77, whereas pigs 78 and 79 showed a gradual decrease over time. As shown in **Figure 10B**, two out of four animals (pigs 84 and 86) of the group immunized with 2 mg of B_2T-TB_2 induced detectable levels of nAbs at day 7 pi (0.9 ± 0.3 log_{10}). By day 14, the nAb titers were slightly higher (1.3 ± 0.2 log_{10}) than those found in the B_2T-3A group, increasing at day

21 pi (1.5 ± 0.2 log_{10}) and remaining constant by day 28 (1.5 ± 0.3 log_{10}) with titers higher than those elicited by B_2T-3A. After boosting, at day 91 pi, pigs 81 (2.7 log_{10}), 84 (2.1 log_{10}) and 86 (2.5 log_{10}) significantly increased nAb titers whereas pig 82 (1.2 log_{10}) did not boost. The overall nAb titers were maintained in subsequent days 127 pi (2 ± 0.4 log_{10}) and 154 pi (1.9 ± 0.5 log_{10}). A similar time course of nAb was observed in pigs immunized with the lower dose of B_2T-TB_2 (0.5 mg), as shown in figure **Figure 10C.** No neutralizing activity was detected at day 7 in any animal. However, detectable nAb were seen at day 14 in pigs 87, 88 and 89 (1.3 ± 0.5 log_{10}). Conversely, pig 90 did not induce significant nAb at this day. The titers increased by day 21 pi (1.5 ± 0.4 log_{10}) and maintained until day 28 pi (1.4 ± 0.4 log_{10}). After the boost, all the animals significantly increased the levels of nAb at day 91 pi (2.0 ± 0.3 log_{10}), a slight decrease was noticed at at day 127 pi (1.8 ± 0.6 log_{10}) and significant titers were maintained until day 154 pi (1.8 ± 0.1 log_{10}). No nAb titers were observed in PBS-inoculated pigs (**Figure 10D**).

When compared, the nAb titers elicited by B_2T-TB_2 at day 28 pi (before boosting) either with 2 mg dose (1.5 ± 0.3 log_{10}) or 0.5 mg (1.4 ± 4 log_{10}) were higher than those of the B_2T-3A group (1.2 ± 0.4 log_{10}). Moreover, focusing on the induction of long-term nAbs, the titers reached at day 154 pi in animals immunized with B_2T-TB_2, either in the 2 mg group (2 ± 0.5 log_{10}) or in the 0.5 mg group (1.8 ± 0.1 log_{10}), were also higher than those induced by B_2T-3A (1.6 ± 0.6 log_{10}). Overall, these results show a trend towards B_2T-TB_2 eliciting nAbs that lasted longer than those evoked by B_2T-3A.

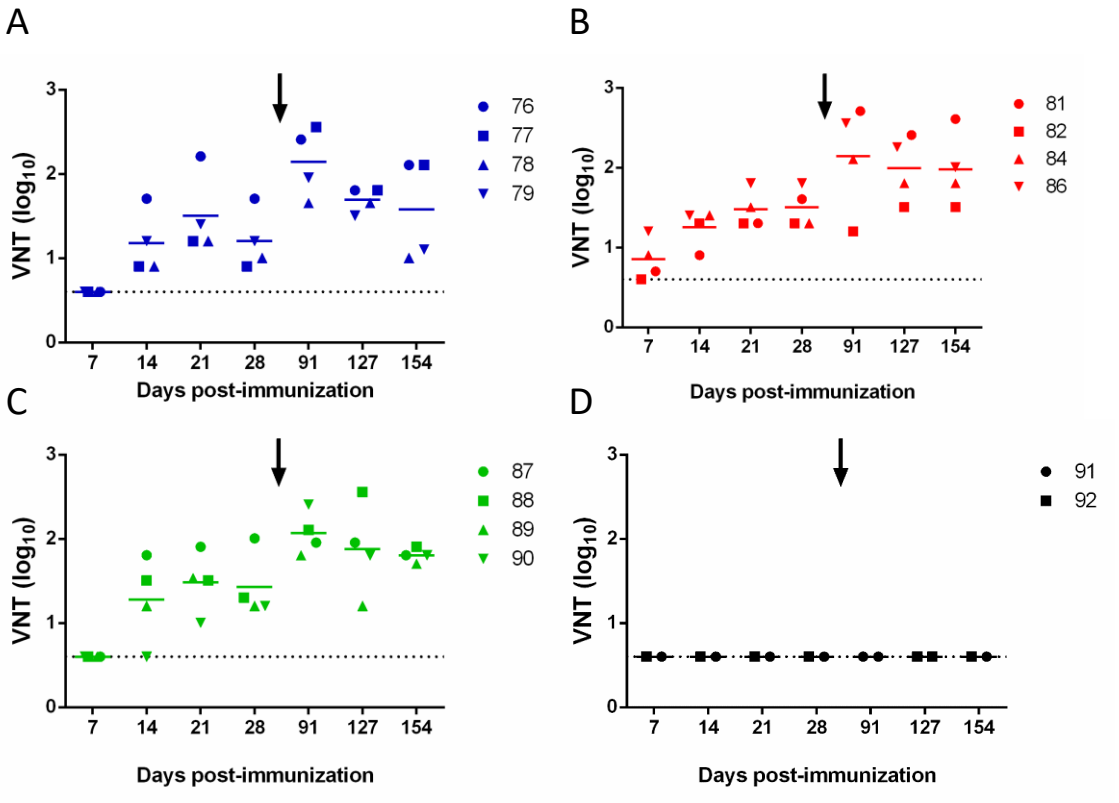


Figure 10: Long-term induction of nAbs in pigs immunized with B_2T-3A and B_2T-TB_2. VN titers in animals immunized twice with B_2T-3A (A), B_2T-TB_2 (2 mg) (B) or 0.5 mg (C) (n=4) or PBS-inoculated (n=2) (D). Virus neutralization titers are expressed as the reciprocal log_{10} of the last serum dilution that neutralized 100 TCID50 of homologous FMDV. Each symbol represents the value for an individual pig. Horizontal lines indicate the geometric mean for each animal group. Arrows show the day of the boost and dotted lines the detection limit.

5.2.3 Detection of isotype-specific antibodies by ELISA

IgG class-switching is determinant to induce protection against different viruses including FMDV. In particular, it has been reported that a low IgG1/IgG2 ratio correlates with protective immune responses in swine. In this line, the ability of the peptides to induce specific IgG1 or IgG2 was analyzed at day 28 pi using an isotype specific ELISA. As shown in **Figure 11A**, B_2T-3A immunized pigs reached similar IgG1 and IgG2 titers (4 log_{10}). Moreover, relative to B_2T-3A, a high dose of dendrimer B_2T-TB_2 (2 mg) induced similar levels of both isotypes (**Figure 11B**). Conversely, two animals of the group immunized with a lower dose (0.5 mg) of B_2T-TB_2, induced slightly less antibody titers of each isotype (around 3 log_{10}), a consistent result taking into account the 4-fold less dose received by these animals (**Figure 11C**). Non-immunized animals did not induce either IgG1 or IgG2 detectable antibodies (data not shown). In summary, no major differences were observed in the IgG1/IgG2 ratio induced by the two dendrimers.

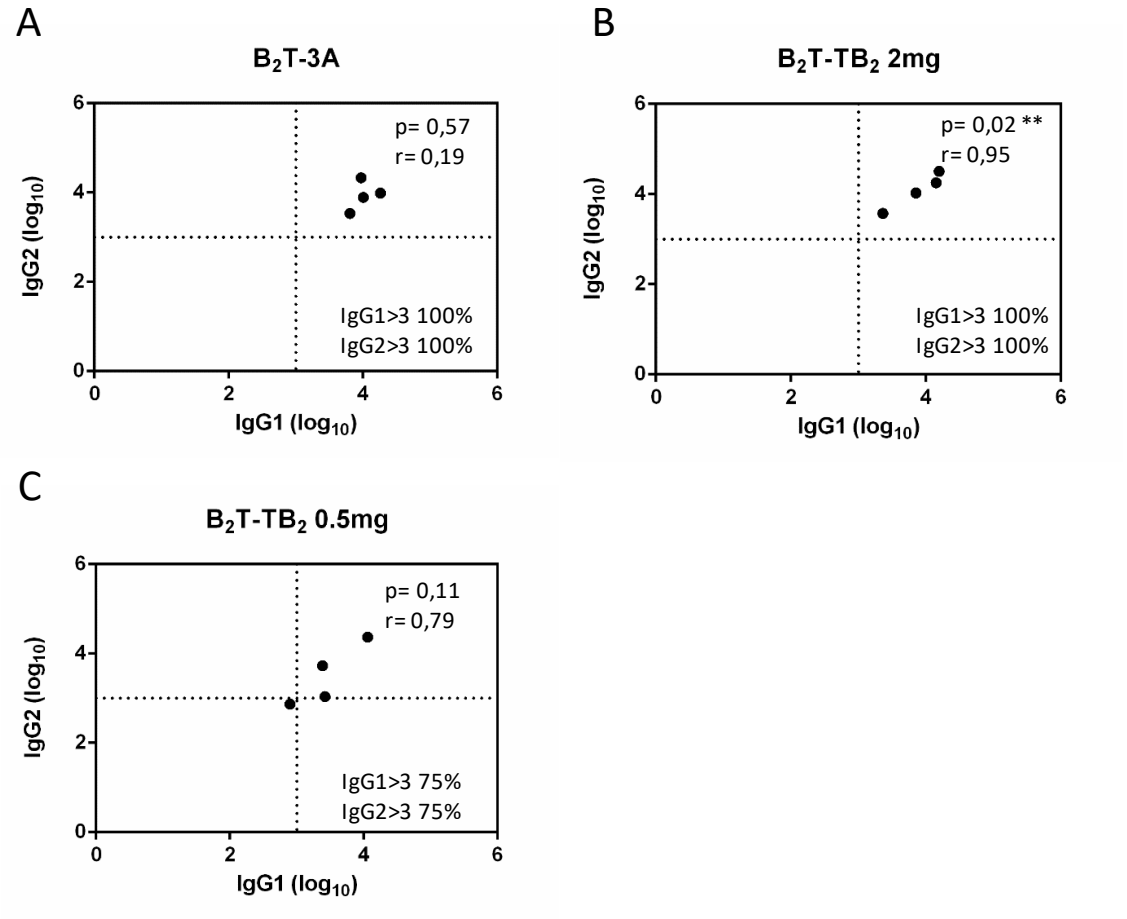


Figure 11: Dendrimers B_2T-3A and B_2T-TB_2 induce similar levels of specific IgG1 and IgG2. Isotype-specific IgG1 and IgG2 antibodies elicited by dendrimers B_2T-3A (A), and B_2T-TB_2 (B) 2 mg or (C) 0.5 mg. Antibodies were detected by ELISA as described in Materials and Methods (see 4.12). Each point represents the value of a duplicate from an individual animal. Endpoint titers are expressed as the reciprocal of serum dilution (log_{10}) giving the absorbance recorded in control wells (sera collected at day 0) + 2 x SD. Each symbol represents the IgG1 and IgG2 titers (X and Y values respectively) for an individual pig. The upper right quadrant shows the coefficient of correlation (*r*) and the p-value (*p*) of each group. The lower right quadrant shows the percentage of pigs with titers above than 3 log_{10}. In no case individual spontaneous reactivity was observed in the titers determined at day 0.

5.2.4 The dendrimers B_2T-3A and B_2T-TB_2 are able to induce specific IFN-γ secreting T-cells

The ability of the dendrimer peptides to induce specific cellular immune responses was determined using an ELISPOT assay to detect IFN-γ secreting cells. PBMCs collected at different days pi from peptide immunized pigs, were *in vitro* stimulated with the homologous dendrimer or the linear T-cell peptide (3A) (hereafter T-3A) for 48 h. In general, animal-to-animal variations were observed in all the groups. In the B_2T-3A immunized pigs, IFN-γ secreting cells were detected in all animals in response to the homologous dendrimer at day 14 pi, and this response was consistent until day 28. Upon the boost, day 91 pi was the first day in which sampling of animals was possible, due to

logistic constrains. At this day, corresponding to day 52 post-boost, specific T-cell responses were still detected and remained until the end of the experiment at day 154 (5 months pi) (**Figure 12**). Similar levels of IFN-γ secreting cells were observed when PBMCs were stimulated with the T-cell epitope T-3A (**Figure 12**). Although the magnitude of the response was lower than that observed with the whole dendrimer, particularly after boost, specific responses were still detected at day 154, mainly for pigs 76 and 78.

In general, the animals immunized with each of the two B_2T-TB_2 doses showed a pattern of T-cell activation similar to that of the B_2T-3A group, being the response to the dendrimer detectable from day 14 pi. After the boost, at day 91, all the animals showed similar levels of IFN-γ expressing cells that were maintained until day 154 pi. As observed in the B_2T-3A group, responses were in general lower when T-3A peptide was used as stimulus, being the difference between the two animals that were good responders and those low responders more marked. Likewise, one of the responder animals immunized with 0.5 mg of B_2T-TB_2, showed a delayed highest response at day 28 (**Figure 12**). After the boost, all the animals maintained their specific T-cell responses. When the T-3A peptide was used for *in vitro* recall, PBMCs from only two pigs in each 2 mg and 0.5 mg group, were stimulated. In none of the non-immunized animals IFN-γ spots were detected in response to these stimuli, confirming the specificity of the response (**Figure 12**).

Overall, no substantial differences were observed among the animals immunized with B_2T-3A and B_2T-TB_2 (2 or 0.5 mg) since the IFN-γ response of PBMCs over time was similar in the three groups in both, the primary and the secondary *in vitro* responses to the peptides.

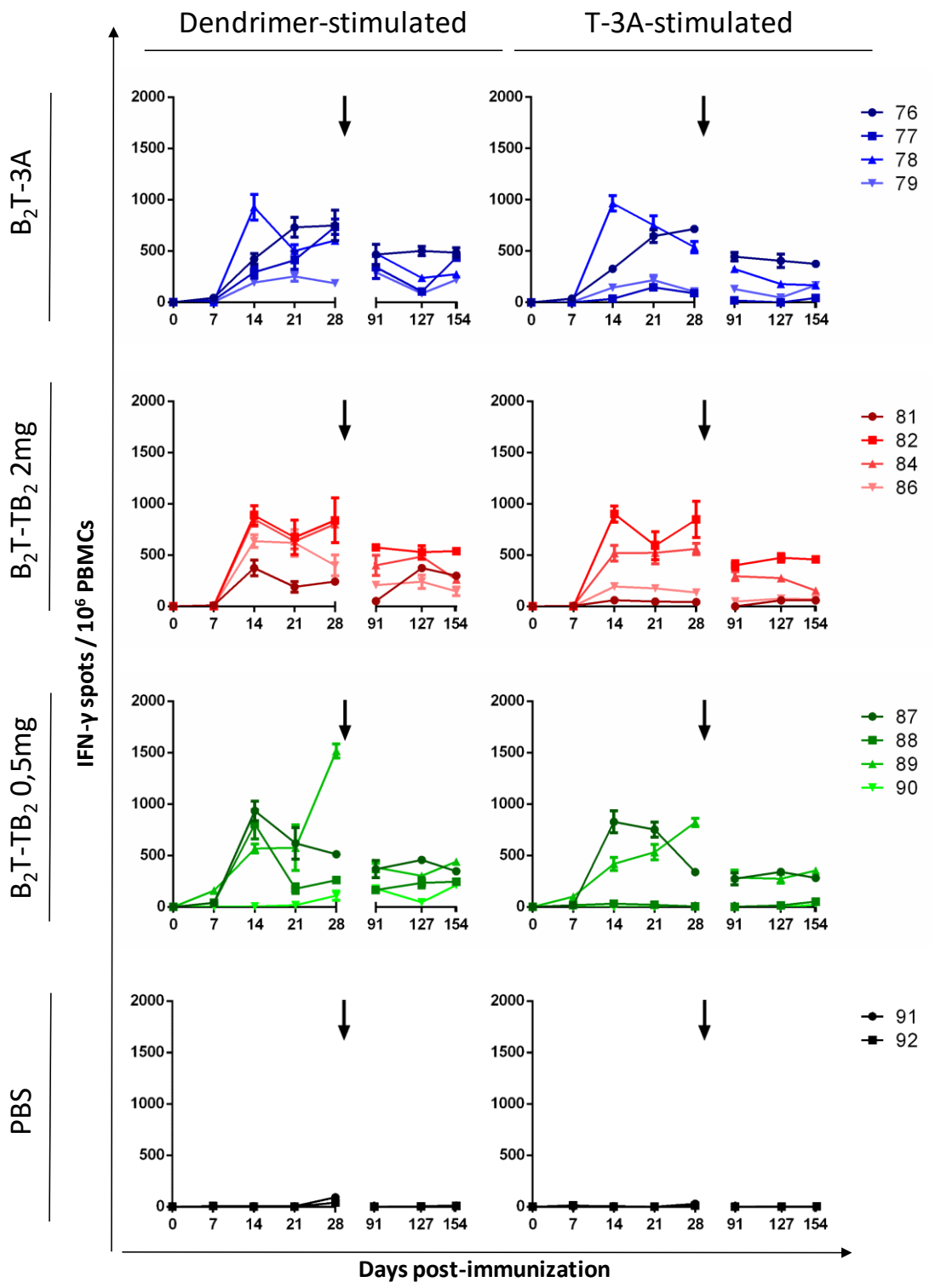


Figure 12: Frequency of specific IFN-γ-secreting cells in PBMCs from pigs immunized with B_2T-3A and B_2T-TB_2 dendrimers. PBMCs from pigs immunized with 2 mg of B_2T-3A, 2 mg of B_2T-TB_2 or 0.5 mg of B_2T-TB_2 or PBS were *in vitro* stimulated for 48 h with the homologous dendrimer or T-3A peptide. The total number of cells expressing IFN-γ was determined from samples collected at different days pi. Mock-stimulated cells were used as a control of the assay and the number of spots recovered from them was considered as background (see Materials and Methods 4.13). Each point-line corresponds to an individual animal and the arrow represents the day of the boost.

5.3 A single immunization with B_2T-3A can confer protection against FMDV challenge

Despite the promising performance of dendrimer B_2T-TB_2 as a FMD vaccine, the limitations inherent to the lack of optimization of its synthesis (see Discussion 6.2.1) led us to focus the subsequent studies on further characterizing the vaccine potential of B_2T-3A.

Efficient vaccination in areas where FMD is circulating often requires vaccines capable to perform as what is so called an emergency vaccine, capable of eliciting protective responses upon a single immunization (see 2.5 in Introduction). The levels of VNT evoked by a single dose of peptide B_2T-3A were in the range of those observed in previous experiments in pigs protected against virus challenge upon receiving two doses of 2 mg each of B_2T-3A [(see 5.2.2) and (Blanco et al., 2016)]. This led us to assess the protective capacity of a single dose of B_2T-3A against homologous viral challenge. FMDV vaccine production needs to be cost-effective. Thus, in a first attempt to explore the feasibility of reducing the dose of B_2T-3A capable of eliciting protective responses, the immunogenicity of two amounts of this peptide was tested. To this end, groups of 5 pigs were given a single injection of a high dose (2 mg) (pigs 1 to 5) or a low dose (0.5 mg) (pigs 6 to 10) of peptide B_2T-3A. Two control animals were non-vaccinated and used as controls (pigs 11, 12). At day 25 pi, all the pigs were challenge with FMDV O/UKG/11/2001 and the protective capacity afforded by B_2T-3A was evaluated. A detailed experimental approach is shown in **Figure 13**.

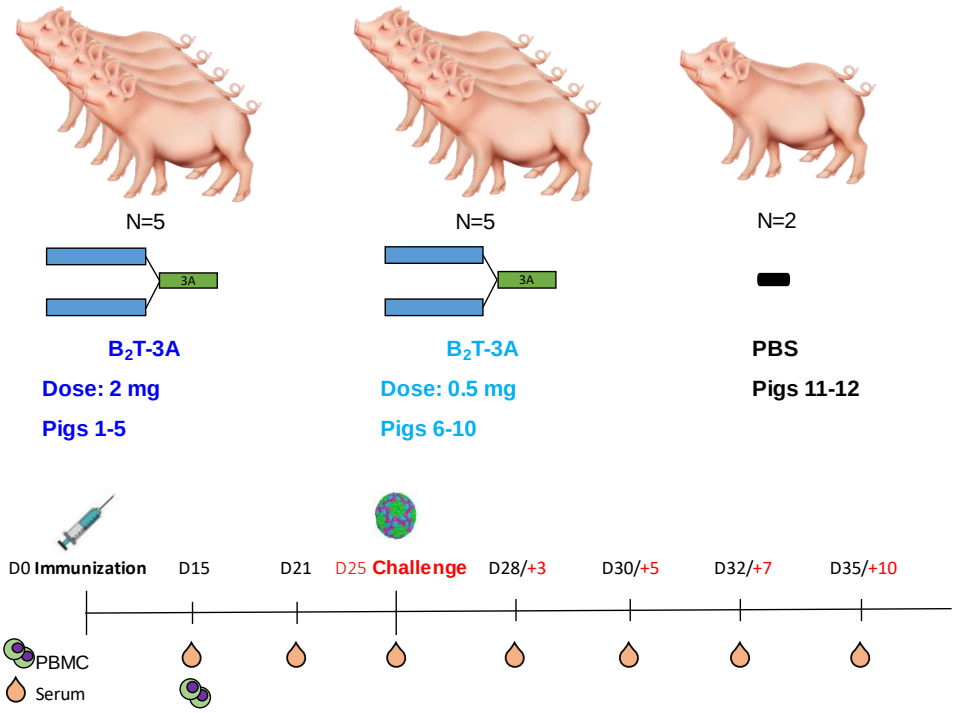


Figure 13: Experimental scheme of the immunization of pigs with a single dose of the dendrimer B_2T-3A and their FMDV challenge. Groups of five pigs were given a high dose (2 mg) or a low dose (0.5 mg) of B_2T-3A. Two additional non-immunized pigs were kept as a controls. At day 25 pi, animals were challenged with 1.6 x 10^4 PFU of FMDV O/UKG/11/2001. Upon challenge, animals were monitory daily for 10 days and sera samples were collected when indicated with a drop. PBMCs were collected at day 15 pi. Days pi are indicated in black and days pc in red.

5.3.1 B_2T-3A elicits a rapid onset of humoral specific responses to FMDV including nAbs

Specific anti-FMDV antibodies were determined by ELISA in sera from pigs at days 0, 15 and 21 pi. Both 2 and 0.5 mg doses of peptide B_2T-3A elicited consistent and comparable IgG titers at days 15 [4.3 ± 0.4 *vs* 3.7 ± 0.3 $\log_{10}$] and 21 [4.2 ± 0.2 *vs* 4.3 ± 0.3 $\log_{10}$] pi (**Figure 14A**). After FMDV challenge these titers were not boosted up, remaining similar in both B_2T-3A immunized groups [4.3 ± 0.1 *vs* 4.6 ± 0.5 $\log_{10}$]. The control non-immunized pig that survived at day 10 pc showed anti-FMDV titers about 2 log units lower than those of B_2T-3A immunized and challenged pigs (**Figure 14A**).

Likewise, significant VNT titers were found at day 15 pi (1.6 ± 0.2 *vs* 1.5 ± 0.4 $\log_{10}$), which slightly increased at day 21 pi (1.9 ± 0.4 *vs* 2 ± 0.6 $\log_{10}$) (**Figure 14B**). As observed with the ELISA results, no major differences were found between animals immunized with each of the two doses of B_2T-3A employed (0.5 and 2 mg). After virus challenge, in contrast with the antibodies detected by ELISA, VNT titers increased around 1 log at day 35 pi (3.1 ± 0.3 *vs* 3.2 ± 0.4 $\log_{10}$). No nAbs were found in the two control animals before

challenge. The non-vaccinated pig that survived the challenge, showed neutralizing titers similar to those of immunized animals (3.1 log_{10}).

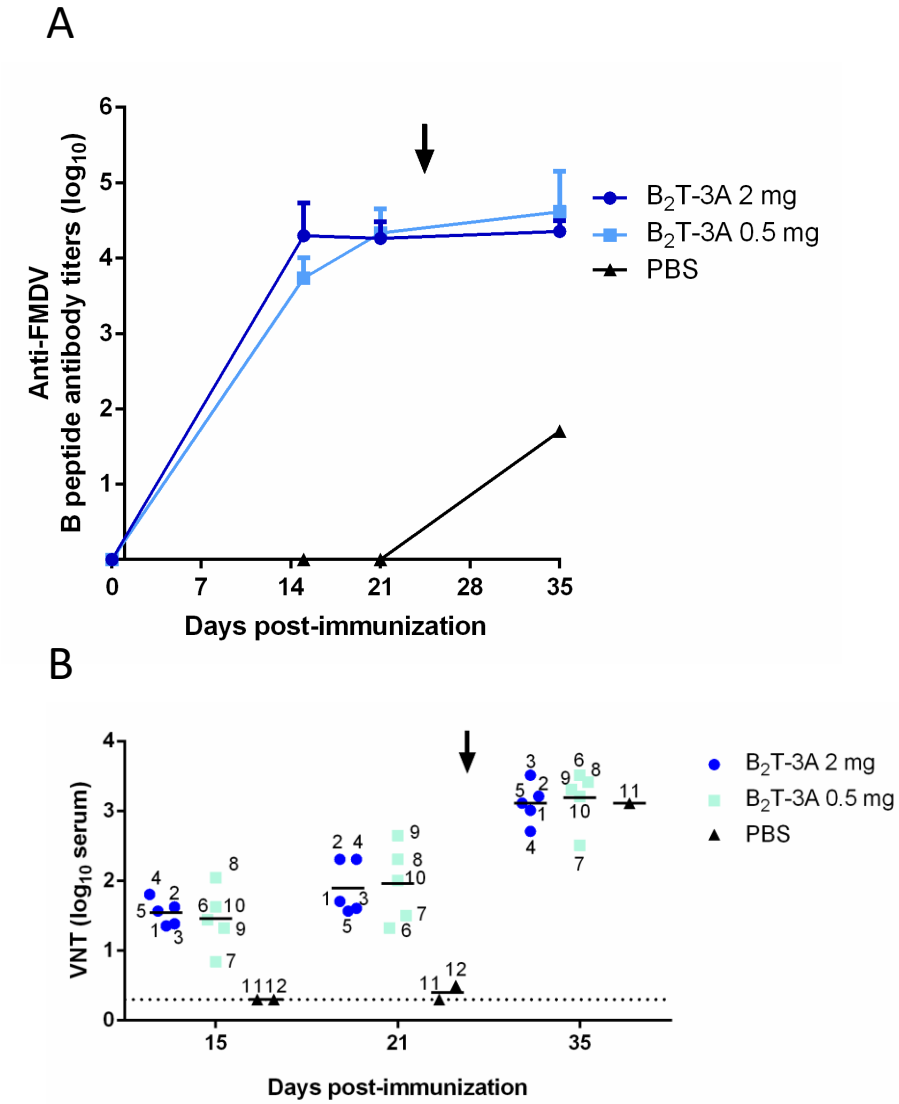


Figure 14: **Similar antibody responses in pigs given a single inoculation with different amounts of B_2T-3A**. The time course of the specific antibody responses to FMDV in sera collected at the days pi indicated. (A) Total IgG antibody response analyzed by ELISA. Each point depicts mean antibody titers (calculated as described in Material and Methods 4.10) ± 2 x SD for each group of pigs (n=5). (B) VNTs expressed as the reciprocal log_{10} of the last serum dilution that neutralized 100 TCID50 of homologous FMDV. Each symbol represents the value for an individual pig. Horizontal lines indicate the geometric mean for each animal group. Arrows show the day of FMDV challenge.

5.3.2 B_2T-3A elicits early FMDV-specific IFN-γ responses

T-cell responses were determined at day 15 pi by ELISPOT of IFN-γ-producing PBMCs. High frequencies of IFN-γ spots were found in both 2 mg and 0.5 mg B_2T-3A immunized pigs in response to *in vitro* recall with homologous B_2T-3A peptide [284.8 ± 321.1 *vs* 645.6 ± 467.9] and with the T peptide [241.3 ± 365.8 *vs* 614.4 ± 403.1] (**Figure 16**). In

average, pigs immunized with 0.5 mg of B_2T-3A showed higher T-cell responses than those with 2 mg of peptide. IFN-γ responses were specific, as no peptide-driven IFN-γ-secreting cells were detected in non-immunized pigs (**Figure 15**).

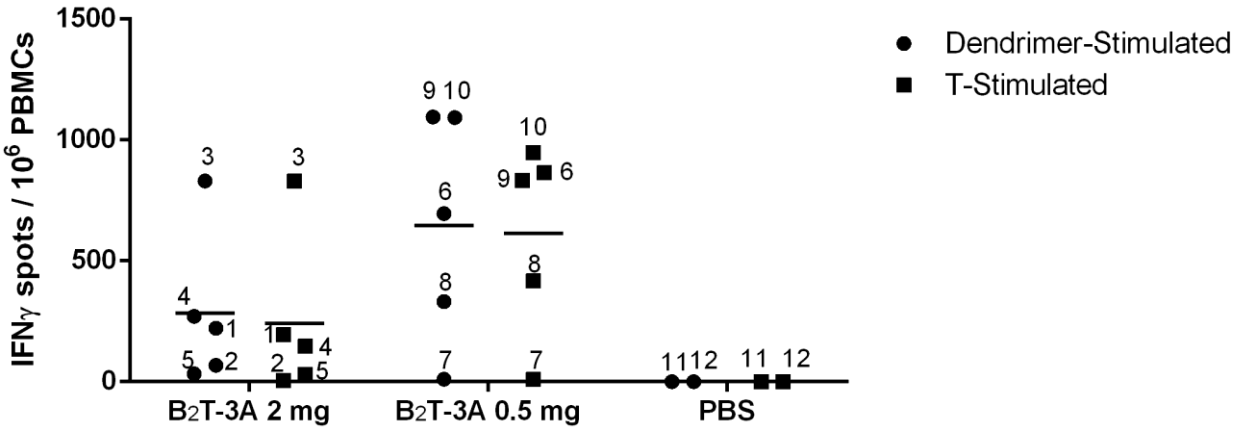


Figure 15: T-cell responses in pigs given a single inoculation with different amounts of B_2T-3A. IFN-γ production at day 15 pi of B_2T-3A (2 mg or 0.5 mg) immunized pigs. Cells were stimulated *in vitro* for 48 h with homologous dendrimer or T-cell peptide. Each point represents the mean of a triplicate of an individual animal (the number of each animal is indicated). Horizontal bars represent the mean of each peptide group (n=5) and the PBS-inoculated control group (n=2).

5.3.3 A single dose of B_2T-3A peptide can confer protection against FMDV challenge

The pigs in the three groups were challenged at day 25 pi with FMDV O/UK/11/01. Animals were examined daily for clinical signs (see Materials and Methods 4.8.2) and considered protected when lesions were not observed or appeared only at the inoculation site. As expected, PBS-inoculated control pigs 11 and 12 showed full FMD signs upon challenge, with vesicular lesions in all four feet by day 4 pc and on the snout also by days 7 and 5 pc, respectively (**Table 5**). The acute FMDV infection caused myocarditis, leading to heart failure and sudden death of pig 12 on day 5 pc. In contrast, only three peptide-immunized animals developed lesions out of the inoculation site, namely pigs 3, 5 and 10, immunized with 2 and 0.5 mg of B_2T, respectively. The remaining immunized animals did not develop clinical signs (**Table 5**).

Thus, immunization with 2 mg and, even more, with 0.5 mg of B_2T-3A dendrimer peptide afforded substantial levels of protection against conventional FMDV challenge: 60% and 80%, respectively.

Table 5: Clinical signs upon FMDV challenge in peptide-immunized pigs

Inoculum	Animal	Fever [a]	Lesion Score[b]	Protected[c]
B_2T-3A (2 mg)	1	39.8 (6)	0	++
	2	39.7 (6)	0	++
	3	41.7 (7)	3 (7)	-
	4	No fever	0	++
	5	39.9 (10)	2 (10)	+
B_2T-3A (0.5 mg)	6	No fever	0	++
	7	No fever	0	++
	8	No fever	0	++
	9	No fever	0	++
	10	39.6 (8)	5 (7)	-
PBS	11	41.7 (6)	7 (7)	-
	12	No fever	7 (5)	-

[a] Temperature (^{0}C) and (in parenthesis) day pc when maximum temperature registered. No fever: ≤39.0-39.5 ^{0}C.
[b] Animals were monitored up to day 10 pc for lesions. Lesion score (maximum value of 7): 1 point/vesicle in foot (up to 4 points); 1 point/mouth, tongue or snout lesion; 1 point/>2 lesions of diameter ≥ 10 mm. In parenthesis, day pc when lesion(s) was first observed.
[c] Animals were considered: fully protected if lesion score ≤ 1 (++); partially protected: mild/delayed disease if lesion score ≤2, or (-) non protected.

5.3.4 Detection of viral RNA in sera upon FMDV challenge

To determine whether the vaccination with peptide B_2T-3A would protect from FMDV replication, detection of viral RNA from samples was analyzed in vaccinated pigs after challenge by RT-qPCR. As shown in **Table 6**, presence of viral RNA was detected in non-immunized control pigs 11 (days 3 and 5 pc) and 12 (day 3 pc), as well as in the two immunized but non-protected pigs 10 (days 3 and 5 pc) and 3 (only at day 5 pc), which showed the higher lesion score at day 7 pc (**Table 6**)

Table 6: Viremia detected in FMDV-challenge pigs after single peptide immunization.

Inoculum	Animal	Day pc[a]				
		0	**3**	**5**	**7**	**10**
B_2T-3A (2 mg)	1	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND
	3	ND	ND	$1.8x10^4$	ND	ND
	4	ND	ND	ND	ND	ND
	5	ND	ND	ND	ND	ND
B_2T-3A (0.5 mg)	6	ND	ND	ND	ND	ND
	7	ND	ND	ND	ND	ND
	8	ND	ND	ND	ND	ND
	9	ND	ND	ND	ND	ND
	10	ND	10^8	$2x10^6$	ND	ND
PBS	11	ND	$1.4x10^8$	$4.5x10^6$	ND	ND
	12	ND	$1.1x10^8$	RIP	RIP	RIP

[a] ND: RNA not detected (detection limit: 100 VRC / ml serum). The values from RNA positive animals are presented as viral RNA copies per ml serum (VRC / ml) (see Materials and Methods 4.15.2). RIP represents the death of the animal.

5.4 Immunogenicity of a B_2T construction harboring a T-cell epitope from FMDV non-structural protein 3D

As commented in Materials and Methods (see 4.5), the 3D epitope (residues 56-70 of 3D protein) had been identified as a potent T-helper epitope in swine (Garcia-Briones et al., 2004). The results obtained in the mouse model showed that different T-cell epitopes previously identified in swine were not efficiently recognized by murine lymphocytes (5.1). Considering this, and despite the limited amount of nAbs elicited by B_2T-3D in mice, we decided to test the effect of the inclusion of this 3D epitope on the immunogenicity of B_2T dendrimer in swine, including the longevity of the response elicited. To this end, as shown in **Figure 16**, groups of four pigs were immunized with 2 mg of B_2T-3A (pigs 80, 81, 82 and 83), B_2T-3D (pigs 84, 85, 86 and 87) or non-immunized (88 and 89). At day 21

pi the animals were boosted with the same amount of peptide and serum and PBMCs samples were collected at different times post-boost.

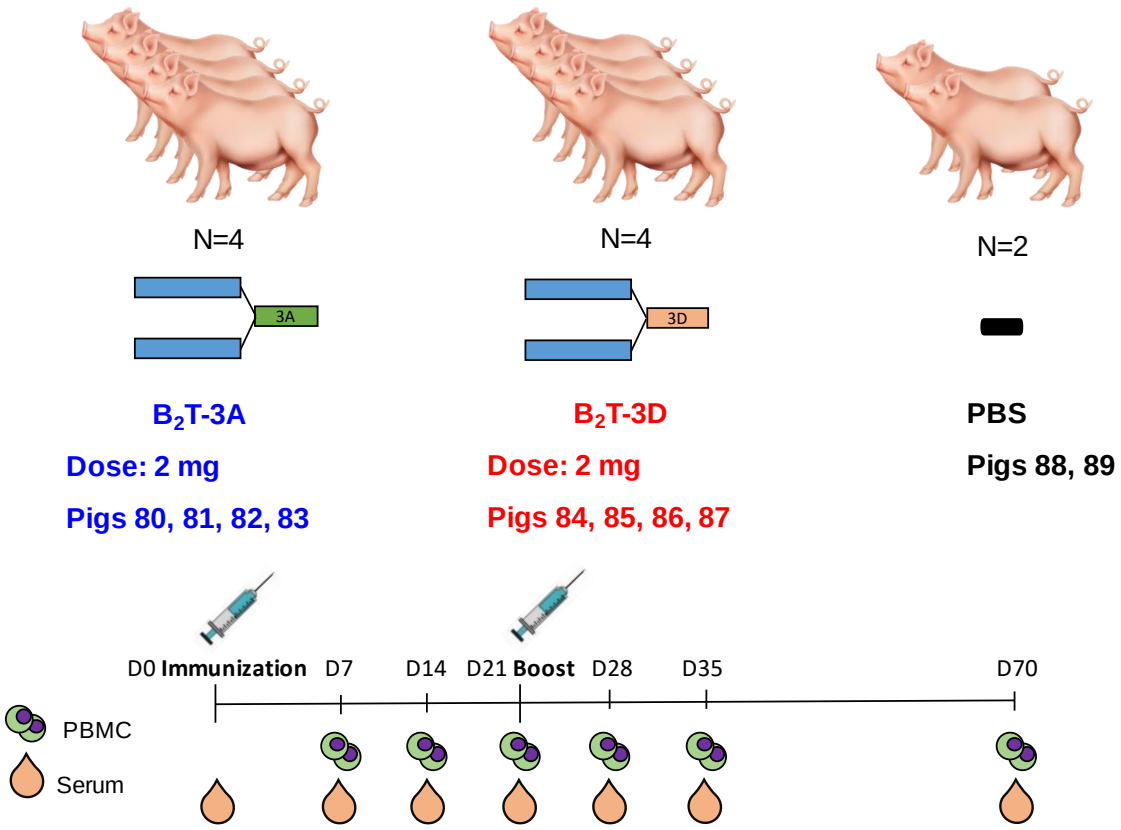


Figure 16: Experimental scheme followed to study the duration of the immunity elicited by B_2T-3D. Groups of four domestic pigs (n=4) were immunized with B_2T-3A (2 mg) or B_2T-3D (2 mg) and boosted at day 21 pi. Sera and PBMCs were collected at the indicated days.

5.4.1 Dendrimers B_2T-3D and B_2T-3A elicit similar antibody responses

The total IgG antibodies elicited by the peptides were measured by ELISA. Specific antibodies were detected in both groups at day 14 pi from which a gradual increment was observed. No remarkable boost effect was observed neither in B_2T-3A nor in B_2T-3D immunized pigs and high levels of IgG antibodies were maintained until day 70 (2 months pi) without significant differences between the two groups. As expected, no specific antibodies were detected in the sera from control PBS-inoculated pigs (**Figure 17A**).

Next, the ability of these antibodies to *in vitro* neutralize homologous virus was tested. B_2T-3A-immunized pigs elicited nAb by day 14 (1.3 ± 0.4 $\log_{10}$) that increased by day 21 (1.6 ± 0.6 $\log_{10}$). After the second peptide dose, the titers increased reaching an average value of 2 ± 0.1 $\log_{10}$ at day 28 pi, and a peak at day 35 pi (2.4 ± 0.1 $\log_{10}$), following a gradual smooth decrease until day 70 pi (1.8 ± 0.4 $\log_{10}$) (**Figure 17B**). Pig 83, did not elicited significant nAb titers following neither the first B_2T-3A dose nor the boost. This

animal was not considered for this comparative study since its health status became deteriorated early during the experiment, showing lameness symptoms and reluctance to eat (**Figure 17B**).

The nAbs from B_2T-3D vaccinated group followed a similar time course and no significant differences were found when compared with B_2T-3A. At day 14 pi, nAbs titers were first observed (1.4 ± 0.1 $\log_{10}$) and increased at day 21 pi (1.8 ± 0.3 $\log_{10}$). After the boost, the average titers reached the peak at day 28 pi (2.1 ± 0.3 $\log_{10}$) and were maintained until day 35 pi (2.1 ± 0.3 $\log_{10}$). A slight decrease, similar to that observed in the B_2T-3A group, was detected at day 70 pi (1.5 ± 0.1 $\log_{10}$) (**Figure 17C**). No neutralizing activity was found in sera from PBS-inoculated animals in any time point (**Figure 17D**)

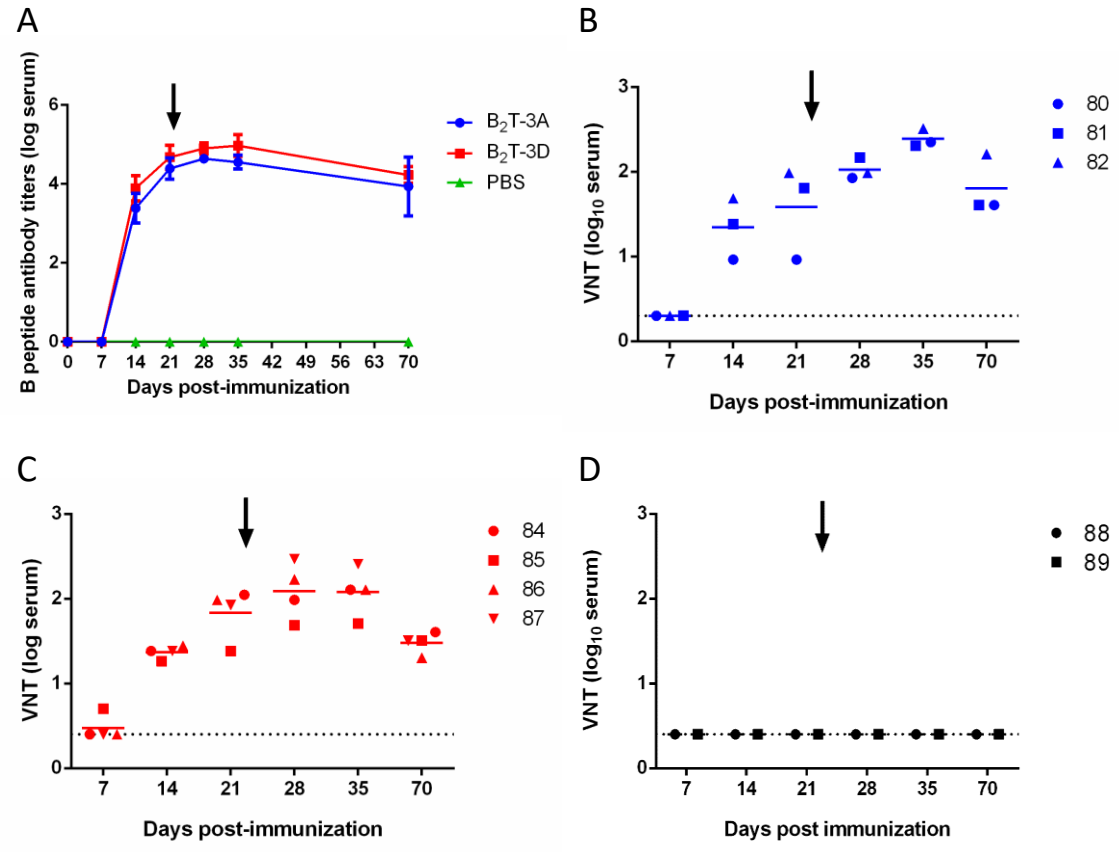


Figure 17: Peptides B_2T-3D and B_2T-3A induce similar antibody responses. (A) Total IgG specific antibody titers measured by ELISA in sera collected at different days pi. Points depict mean antibody titers for each group of pigs. VNT in sera from animals immunized with (B) B_2T-3A, (C) B_2T-3D and (D) non-immunized. Titers are expressed as the reciprocal $\log_{10}$ of the last serum dilution that neutralized 100 TCID50 of homologous FMDV. Each symbol represents the value for an individual pig. Horizontal lines indicate the geometric mean for each peptide-inoculated animal group (n=4) and dotted lines the detection limit. Arrows show the day of the boost.

5.4.2 T-cell responses elicited by B_2T-3D and B_2T-3A

The capacity of peptide B_2T-3D to induce specific T-cell responses was assessed in PBMCs isolated from immunized pigs by ELISPOT analysis of the IFN-γ-secreting cells. In this experiment, the B-cell peptide was included as stimulus for the *in vitro* recall, to address the possibility of its recognition by immune T-cells. As in previous experiments, intragroup variability was observed in the responses, which was reflected in the presence in each group of high responders (B_2T-3A: pigs 81 and 82; B_2T-3D: pigs 86 and 87) and low responders (B_2T-3A: pigs 80; B_2T-3D: pigs 84 and 85). A remarkable primary response of IFN-γ secreting cells was noticed at day 14 pi (**Figure 18**). Interestingly, the two high responders pigs in the B_2T-3D group showed more IFN-γ spots than the higher responders in the B_2T-3A group when their PBMCs were stimulated with the whole homologous dendrimer (1743 ± 364 for B_2T-3D group *vs* 1206 ± 244 for B_2T-3A group) and the specific T-cell epitope (1679 ± 453 *vs* 959 ± 587). The magnitude of the responses was lower when cells were stimulated with the B-cell peptide, being the values higher in pigs immunized with B_2T-3A (130 ± 97 *vs* 507 ± 183) (**Figure 18**). At day 21 pi the response weaned in both groups reaching similar levels of IFN-γ spots when cells were stimulated with the dendrimer (847 ± 105 *vs* 800 ± 224) and the T-cell epitope (785 ± 5 *vs* 646 ± 382). At this time, responses against peptide B were clearly lower (42 ± 16 *vs* 286 ± 186) (**Figure 18**). After the boost, a non-immediate secondary response was observed at day 35 pi in the two major responders in each group when stimulated with the corresponding dendrimer (1073 ± 132 *vs* 1161 ± 87, respectively). However, when stimulated with the T-cell epitope, the IFN-γ production was higher in the B_2T-3D group (929 ± 242 *vs* 637 ± 52). The response dramatically weaned in both groups being scarcely detected at day 70 pi. As expected, non-immunized animals did not induce IFN-γ secreting cells upon stimulation with any of the specific peptides (**Figure 18**).

Interestingly, the frequencies of IFN-γ spots in response to the B-cell peptide in pigs from the B_2T-3D group were considerably lower than those of B_2T-3A immunized animals. This results suggests that the B-cell epitope plays a minor role in cytokine production in B_2T-3D immunized pigs and that T-3D epitope is a more potent inducer of IFN-γ-producing cells than T-3A.

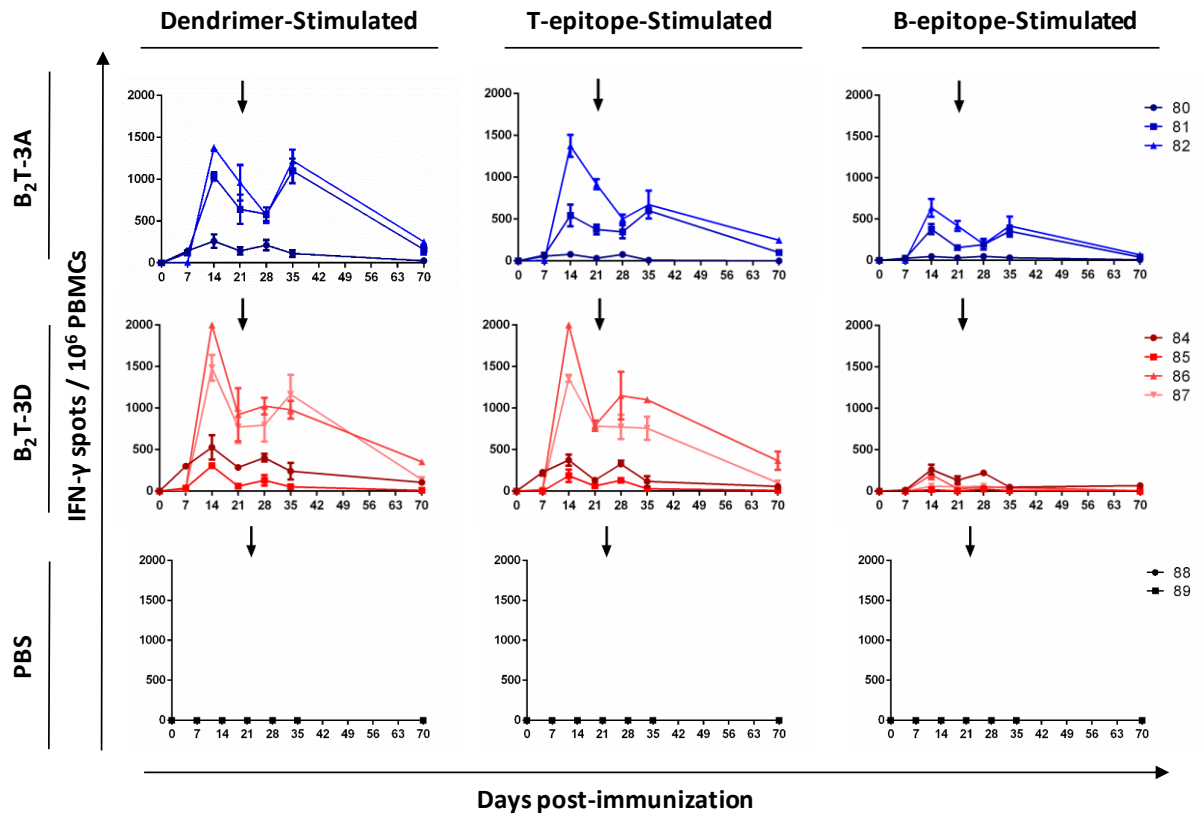


Figure 18: T-cell responses in pigs immunized with B_2T-3A and B_2T-3D constructions. PBMCs isolated from individual animals of each group were collected at different days pi. Cells were stimulated *in vitro* for 48 h with homologous dendrimer, T-cell epitope or B-cell epitope and the number of cells expressing IFN-γ was measured by ELISPOT. PBMCs stimulated with medium (not shown) were included as a negative control of the assay. Each point represents the mean of a triplicate of an individual animal. Arrows show the day of the boost.

5.5 Analysis of the immune response induced by B_2T dendrimers displaying two T-cell epitopes in tandem

The results described in the previous section with the dendrimer B_2T-3D raised the question of whether including both 3A and 3D T-cell epitopes in the same molecule would modulate and how the immunogenicity of the dendrimer. As already mentioned, the MHC restriction can represent a bottleneck in the development of subunit vaccines, since recognition of different T-cell epitopes may vary among individuals of species in which potential wide repertoires of MHC molecules exist (see 2.5.6). For this reason, expanding the repertoire of T-cell epitopes included in dendrimeric vaccines may allow a wider and more efficient recognition and presentation of dendrimers to T-cells from genetically different individuals. Previous results in our laboratory had shown that not only the inclusion but also the orientation of the T-cell epitope influenced the immune response elicited by linear FMDV peptides (Blanco et al., 2013). For this reason, T-cell epitopes T-3A and T-3D were combined in a B_2T dendrimer in the two possible

orientations, giving rise to peptides B_2T-3A3D and B_2T-3D3A. To assess the immunogenicity of these constructs, an experimental approach similar to that in section 5.4, was followed (**Figure 19**). Briefly, groups of four pigs were immunized with B_2T-3A (pigs 49, 54, 65 and 74), B_2T-3D (pigs 75, 76, 77 and 78), B_2T-3A3D (pigs 79, 90, 91 and 92) or B_2T-3D3A (pigs 93, 94, 95 and 96).

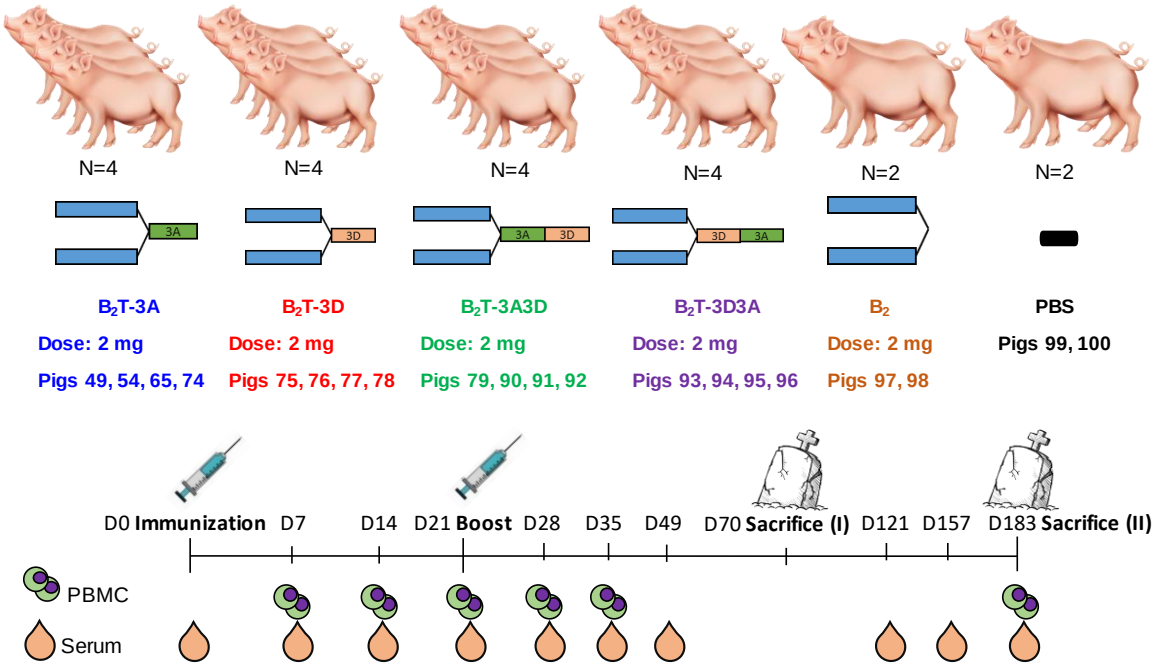


Figure 19: Experimental scheme followed to study the immunity elicited by B_2T constructions combining T-cell epitopes and its duration. Groups of four domestic pigs were immunized with B_2T-3A, B_2T-3D, B_2T-3A3D, B_2T-3D3A. In addition, two pigs were immunized and boosted with B_2. Two non-immunized pigs were included as controls. Because of animal welfare and space requirements, half of the animals (10) corresponding to the lower T-cell responders to the immunizing peptide, were euthanized at day 70 pi. The remaining pigs (10) were housed until the end of the experiment at day 183 pi. Sera and PBMCs were collected at the indicated days.

Previous studies with linear peptides had shown that the inclusion of a T-cell epitope was required for inducing optimal immune responses in pigs (Cubillos et al., 2012). Nevertheless, the requirement for a T-cell epitope in the context of a more complex molecule such as a dendrimer peptide, remained to be clarified. To test the importance of the T-cell peptide in the B_2T constructions, two additional pigs were immunized with peptide B_2 (pigs 97 and 98) that keeps peptide B but lacks any additional porcine epitope. As a negative control, two mock-immunized animals were included (pigs 99 and 100). At day 21 pi the animals were boosted and serum and PBMC samples were collected at different times post-boost (**Figure 19**). Since minimal accommodation space requirements were needed for animal welfare, at day 70 pi, half of the animals that were low responders in the ELISPOT of IFN-γ-secreting cells, were euthanized.

As previously shown (**Figure 18**), T-cell responses to B_2T constructions showed a trend towards decreasing over time, being more short-lived than antibody responses. For this reason, the frequency of IFN-γ-secreting cells was only analyzed early times pi (days 7, 14 and 21) and post-boost (days 28 and 35), and by the end of the experiment at day 183 (6 months pi) for those animlas that remained alive (**Figure 19**).

5.5.1 Similar levels of total IgG antibodies are elicited by dendrimers harboring one or two T-cell epitopes

The total IgG antibodies in sera from peptide immunized pigs were determined by ELISA. All the B_2T-immunized animals elicited similar levels of specific antibodies that were first detected at day 14 pi (about 3.6 $\log_{10}$) and slightly increased at day 21 pi (about 4 $\log_{10}$). Upon the boost, an increment in IgG antibody titers was observed in the B_2T-immunized groups at day 28 pi (about 5 $\log_{10}$) that was maintained up to day 49 pi, except those of pigs immunized with B_2T-3D3A whose titers showed a slight decrease. At day 121 (4 months pi) a sustained antibody response was still observed, with average titers about 4.5 $\log_{10}$. Interestingly, the antibody response was long-lasting and significant titers were detected at the end of the experiment at day 183 (6 months pi) (about 4 $\log_{10}$). No antibodies were detected in B_2 and PBS-immunized groups (**Figure 20**).

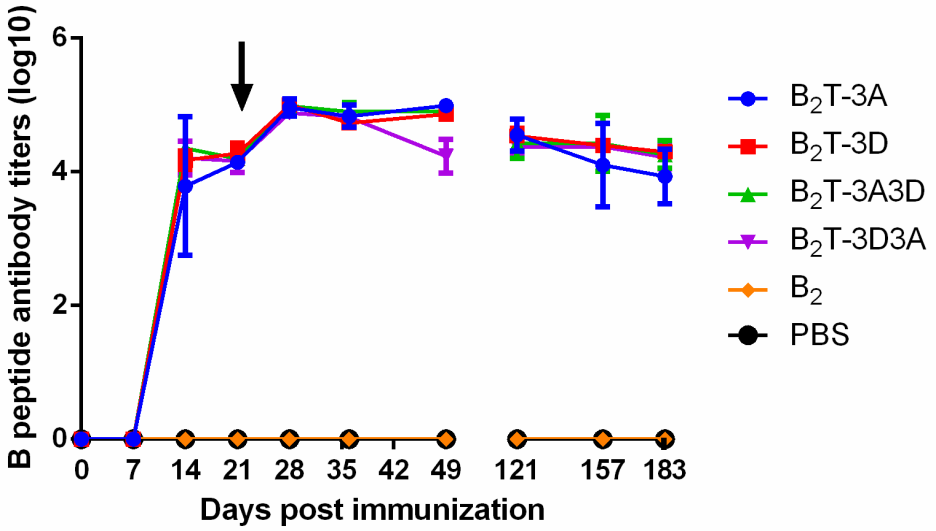


Figure 20: Time course of specific IgG antibodies elicited by B_2T constructions combining different T-cell epitopes. Each line represents the mean of sera from the indicated group at different days pi (n=4; except for B_2 and PBS groups in which n=2) ± SD. Data on day 121 pi correspond to 2 pigs per group. Arrow points the day of boost.

5.5.2 The presence of T-cell epitopes in the B_2T construction is essential for the induction of nAb

When the capacity of the antibodies elicited by the B2T constructions to *in vitro* neutralize homologous virus was tested, no nAbs were detected at day 7 pi (**Figure 21A**), being first detected at day 14 pi. At this time, slightly higher VNT were observed for constructions encompassing two T cells in tandem (B_2T-3A: 1.3 ± 0.6; B_2T-3D: 1 ± 0.4; B_2T-3A3D: 1.6 ± 0.5; B_2T-3D3A: 1.5 ± 0.5 $\log_{10}$) (**Figure 21B**). At day 21 pi, a general increment was observed in all dendrimer-immunized groups, which reached similar average titers (B_2T-3A: 1.9 ± 0.3; B_2T-3D: 1.9 ± 0.4; B_2T-3A3D: 1.6 ± 0.7; B_2T-3D3A: 1.7 ± 0.5 $\log_{10}$) (**Figure 21C**).

After the boost, at day 29 pi, an increase in the VNT was noticed in the pigs immunized with B_2T-3A (2.5 ± 0.6 $\log_{10}$), B_2T-3D (2.5 ± 0.4 $\log_{10}$) and B_2T-3A3D (2.6 ± 0.3 $\log_{10}$). Such a boost effect was not clearly observed in the B_2T-3D3A group (2 ± 0.6 $\log_{10}$) (**Figure 21D**). The titers were maintained over time with minor differences among groups in the subsequent days 36 pi (B_2T-3A: 2.4 ± 0.5; B_2T-3D: 2.5 ± 0.5; B_2T-3A3D: 2.5 ± 0.3; B_2T-3D3A: 1.9 ± 0.5 $\log_{10}$) (**Figure 21E**) and 49 pi (B_2T-3A: 2.3 ± 0.4; B_2T-3D: 2.1 ± 0.6; B_2T-3A3D: 2.2 ± 0.3; B_2T-3D3A: 1.6 ± 0.3 $\log_{10}$) (**Figure 21F**). These differences were not statistically significant. Remarkably, no nAbs were detected in pigs immunized with B_2 peptide at any time point.

These results suggested that the presence of T-cell epitopes and their orientation within the dendrimer can have an impact in the neutralizing memory responses towards the FMDV G-H loop (peptide B). Indeed, when the animals that were maintained and continued the experiment −pigs 49 and 65 (B_2T-3A), 76, 77 and 78 (B_2T-3D), 91 and 92 (B_2T-3A3D), 93 and 94 (B_2T-3D3A)− were analyzed at days 121, 157 and 183 (4, 5 and 6 months pi, respectively) VNT similar to those at day 49 pi were found (data not shown). This result is consistent with the fact that the dendrimers elicit long-lasting FMDV neutralizing responses (see 5.2.2).

Remarkably, the lack of induction of nAbs in the pigs inoculated with the construction B_2, lacking a porcine T-cell epitope while keeping the B-cell epitope, confirm the functional role of T-3A and T-3D as T helper epitopes, as inclusion of at least one of them in the dendrimer construction is necessary to elicit nAbs in pigs.

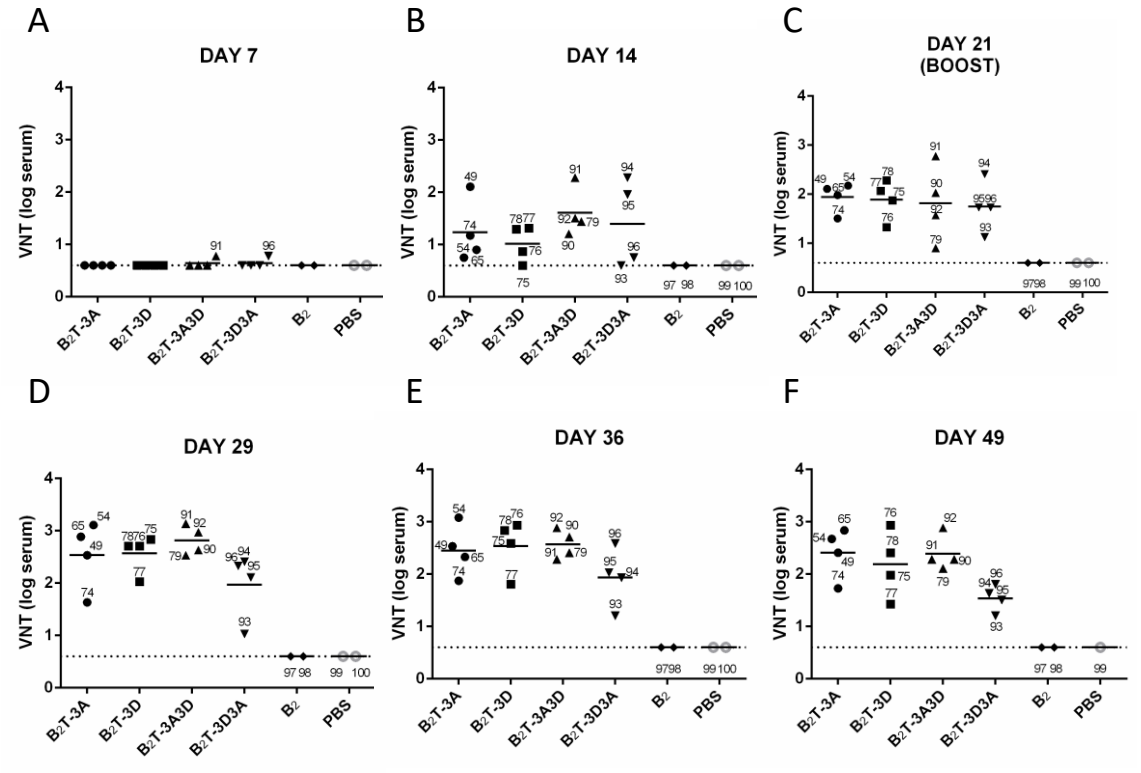


Figure 21: The presence and orientation of the T-cell epitopes in the dendrimer influence the boosting effect of neutralizing antibodies. Sera samples were collected after one dose of each dendrimeric peptide (pre-boost) (panels A, B and C) and after peptide boost (panels D, E and F). Each point represents the VNT of one single pig and the mean of each group is shown with a horizontal bar. Dotted line depicts the detection limit.

5.5.3 Isotype specific IgG1 and IgG2 antibodies elicited by dendrimers B_2T-3A3D and B_2T-3D3A

To test whether the inclusion of two consecutive T-cell epitopes in the dendrimer and its orientation could lead preferentially towards expression of a specific IgG isotype and influence the class switching, the anti B-peptide IgG1 and IgG2 profile was analyzed by ELISA in samples from pigs immunized with the different dendrimer constructions at day 21 pi. All the dendrimers induced specific IgG1 and IgG2 albeit to different extents. These differences were consistent with the magnitude of the total IgG responses recorded above (see 5.5.1). At day 21 pi, three animals from the B_2T-3A group showed high titers of both IgG1 and IgG2 (about 5 $\log_{10}$) and only one pig did not develop significant IgG2 titers (< 3 $\log_{10}$) (**Figure 22A**). All the animals immunized with B_2T-3D reached similar IgG1 and IgG2 titers (about 4 $\log_{10}$) of a magnitude lower than those of the B_2T-3A group (**Figure 22B**). Moreover, pigs immunized with B_2T-3A3D reached isotype titers similar to those of the B_2T-3D group suggesting that T-3D was modulating the isotype switching (**Figure**

22C). On the other hand, the construction B_2T-3D3A induced lower IgGs titers, in particular IgG2 that was detected in 2/4 animals (**Figure 22D**).

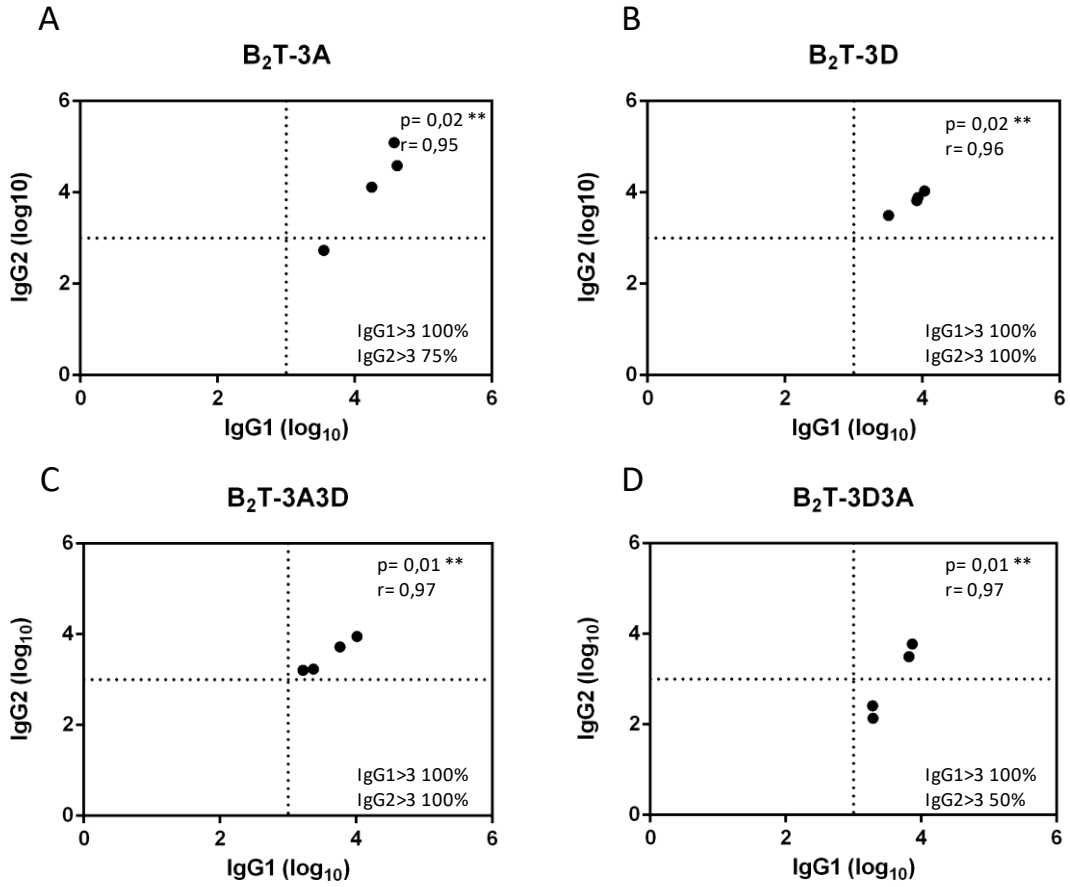


Figure 22: IgG1 and IgG2 profiles elicited by B_2T constructions combining different T-cell epitopes. Anti-B peptide IgG1 and IgG2 profiles in sera from pigs collected at 21 days pi with: B_2T-3A (A), B_2T-3D (B), B_2T-3A3D (C) and B_2T-3D3A (D). Each point represents the value of a duplicate of an individual animal. Endpoint titers are expressed as the reciprocal of serum dilution (log_{10}) giving the absorbance recorded in control wells (sera collected at day 0) + 2 x SD. Each symbol represents the IgG1 and IgG2 titers (X and Y values respectively) for an individual pig. The lower right quadrant shows the percentage of pigs with titers above than 3 log_{10} (significant). In no case individual spontaneous reactivity was observed in the titers determined at day 0.

5.5.4 T-cells responses elicited by B_2T-3A3D and B_2T-3D3A: immunodominance of the 3D T-cell epitope

The T-cell responses elicited by dendrimers B_2T-3A, B_2T-3D, B_2T-3A3D, B_2T-3D3A and B_2 were analyzed using PBMCs collected at different days pi (see **Figure 19)**. Cells were *in vitro* stimulated with the homologous peptide as well as with other dendrimers in an attempt to understand the antigenic specificity of the responses elicited. As observed in previous experiments (see 5.2.4. and 5.4.2.), significant frequencies of IFN-γ expressing cells were detected in the B2T-3A group upon *in vitro* recall with the homologous dendrimer. In general, responses were higher in 3 out of 4 animals (pigs 49,

65 and 74). The number of IFN-γ secreting cells detected was stimulus-dependent, being the higher values induced by the homologous dendrimer B_2T-3A. Responses to T-3A were also significant confirming that this peptide is recognized as a T-cell epitope. A heterologous response against B_2T-3D peptide was also noticed, suggesting that the configuration of the dendrimer and/or the B-cell peptide this peptide contains, may play a role in T-cell activation. The specificity of the T-cell response was confirmed as no IFN-γ spots were detected upon stimulation with the heterologous T-cell epitope, the peptide T-3D. As previously observed (see 5.4.2), IFN-γ secreting cells were also detected upon B-peptide-stimulation, supporting that this B-cell epitope may contribute to the effector cellular responses (**Figure 23A**).

Peptide B_2T-3D was also able to induce dendrimer-specific T-cell responses in all the animals, supporting our previous results (see 5.4.2). Cross-stimulation with B_2T-3A and, to a more extent, with peptide T-3D was observed, confirming that this epitope can be considered as a T-cell activator as well as an effector epitope. As observed in animals immunized with B_2T-3A, the B-cell peptide was also able to induce IFN-γ release, suggesting again that this peptide may be recognized as a T-cell epitope (**Figure 23A**).

When T-3D and T-3A were joined together in dendrimer B_2T-3A3D a strong T-cell response was observed in the pigs immunized with this construction. Notably, the response was higher when cells were stimulated with peptide T-3D rather than with peptide T-3A, suggesting an immunodominance of T-3D over T-3A. A similar effect was noticed when the orientation of the T-cell epitopes was change in dendrimer B_2T-3D3A. All the animals elicited strong responses of IFN-γ secreting cells in response to homologous and heterologous dendrimer. When individual T-cell peptides were used as stimuli, responses were remarkably higher to T-3D than to T-3A. Again, the B-peptide was able to induce IFN-γ spots (**Figure 23A**).

Interestingly, low levels of IFN-γ expressing cells were detected in animals immunized with peptide B_2 that does not contain a porcine T-cell epitope, supporting the contribution of the T-cells on the responses. On the other hand, the cross-stimulation observed in response to B_2T-3D further evidence of the capacity of the B_2 construction and for the extension of the B-cell peptide, to elicit porcine T-cells that recognize the compete dendrimers B_2T-3A and B_2T-3D.

In general, very low levels of IFN-γ secreting cells were noticed when the B-cell peptide was used as stimulus for *in vitro* recall of cells from pigs immunized with B_2T-3A3D or B_2T-3D3A, which was in contrast with the responses induced in the B_2T-3A and B_2T-3D groups (**Figure 23A**). The specificity of the responses detected was confirmed by the lack of stimulation of IFN-γ secreting cells in PBMCs from pigs immunized with peptide B_2 (encompassing only the B cell peptide) in response to individual T-cell peptides. As expected control non-immunized pigs did not elicit specific T-cell responses against any peptide.

To gain information on whether the induction of T-cell responses could be influenced by non-specific mechanisms, a dendrimer with a scrambled amino acid composition at both the B- and the T-3A epitopes (B_2scr-Tscr) was used to stimulate PBMCs from day 183 (**Figure 23B**). Interestingly, no IFN-γ was detected neither in response to the whole scrambled peptide nor to the construction with only the scrambled T-cell.

Altogether, the stimulation observed with the homologous dendrimers was significant and in the same range than those detected in previous experiments, suggesting that the T-cell responses elicited by the dendrimer peptide were sequence-specific. The results also point to the recognition of the T cell epitopes T-3A and/ or T-3D as the main component in the induction of the T-cell responses elicited by B_2T peptides leading to the expression of IFN-γ, a cytokine with a relevant role in the antiviral response (McCullough and Sobrino, 2004).

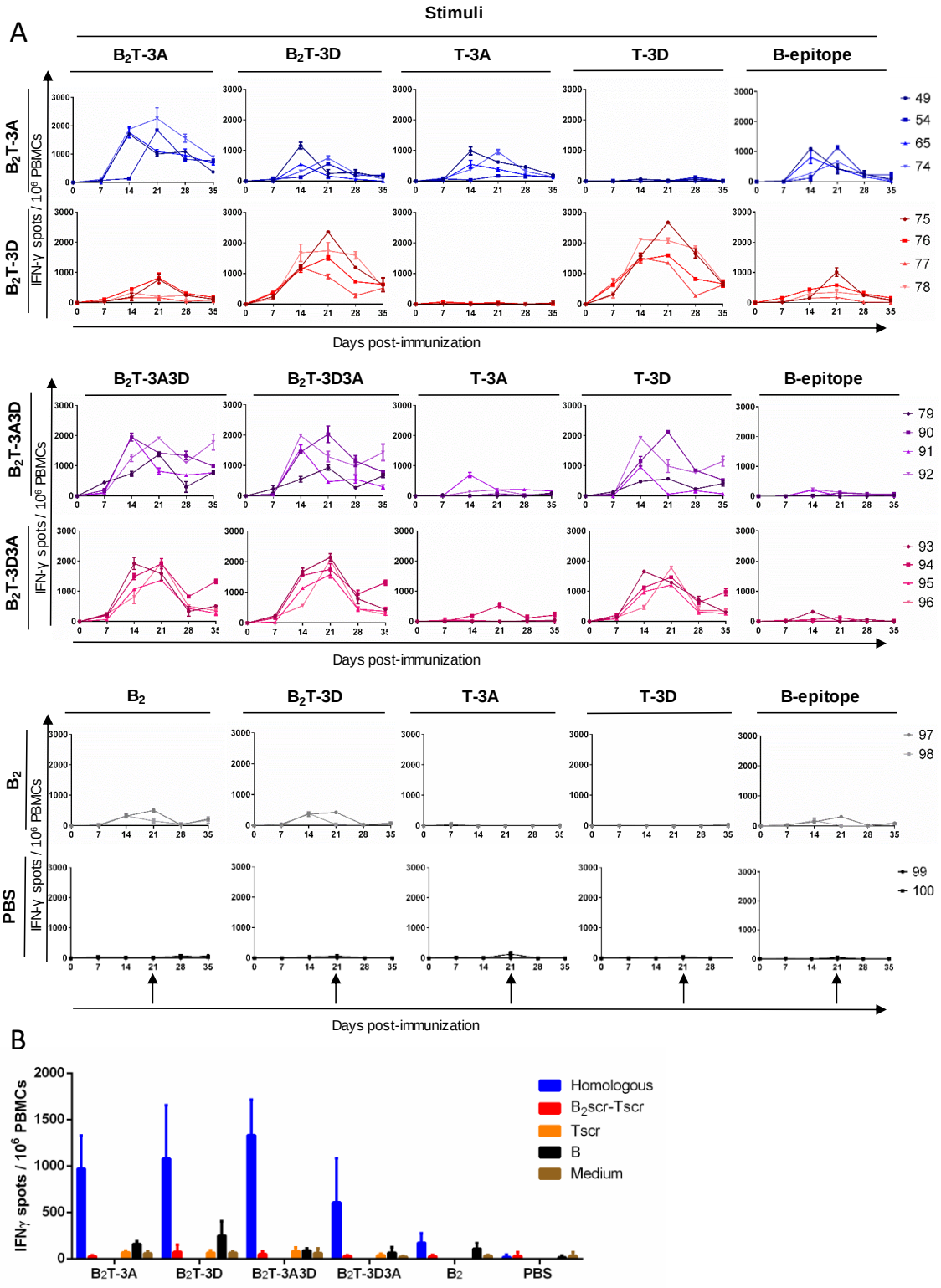


Figure 23: T-cell responses elicited by B_2T-3A3D and B_2T-3D3A unveils 3D peptide as an immunodominant T-cell epitope. PBMCs isolated from individual animals of each group were collected at different days pi. Cells were stimulated *in vitro* for 48 h with dendrimer and the frequency/number of IFN-γ producing cells was measured by ELISPOT. A) Frequency of IFN-γ secreting cells in the groups of pigs immunized as indicated, in response to the homologous dendrimer and the different constructions indicated. Each line corresponds to an individual animal and arrows show the day of the boost. B) The IFN-γ production was stimuli-specific and sequence-dependent. The values are presented as IFN-γ spots per 10^6 cells (spots from mock-stimulated cells are subtracted). Each column represents the mean (n=4) ± SD.

5.6 Characterization of T-cell populations elicited upon immunization with B_2T dendrimers

5.6.1 Flow cytometry analysis unveils $CD4^+$ T-cells as the major population involved in T-cell immunity

In order to evaluate the specific T-cell populations stimulated upon immunization with B_2T dendrimers, PBMCs collected from immunized pigs at day 21 pi were stimulated or not *ex vivo* with homologous dendrimers. To prevent the cytokine release to the extracellular media, the samples were incubated prior to cell staining with brefeldine A (BFA; a drug that disrupts the Golgi complex and inhibits the extracellular pathway) to allow accumulation of the induced cytokines within the cell. Next, the cells were stained with CD4 (helper) and CD8-β (cytotoxic) surface markers using different mAbs and multi-colour staining strategies (see **Table 4**). After surface staining, the cells were permeabilized and intracellular staining was performed to detect IFN-γ expression using different mAb (see 4.14). To make the assay feasible, the two higher responders of each group were selected: pigs 49 and 65 (B_2T-3A), pigs 76 and 77 (B_2T-3D), pigs 91 and 92 (B_2T-3A3D), pigs 93 and 94 (B_2T-3D3A), pig 98 (B_2) and pig 100 (PBS). Control pig 100 was stimulated with PHA as a positive control of the assay (not included in the graph).

Lymphocytes were gated for the expression of $CD4^+$ and $CD8\beta^+$. The percentages of both populations were variable among the pigs (17.4 ± 3.7 % and 10.1 ± 3.2 % respectively) (**Figure 24A**). Within both populations, IFN-γ expression was analyzed. Upon specific stimulation, IFN-γ was preferentially expressed by $CD4^+$ T-cells in all immunized pigs (n=2). The percentage of $CD4^+IFN\text{-}\gamma^+$ cells were variable, being higher in B_2T-3D group (5.2 ± 0.3 %), following B_2T-3A3D (3.6 ± 1.9 %), B_2T-3D3A (3 ± 1.1 %) and B_2T-3A (2.8 ± 2 %). Noteworthy, the B_2-immunized pig (pig 98) hardly induced $CD4^+IFN\text{-}\gamma^+$ T-cells (0.4 %), supporting that the inclusion of a T-cell epitope is necessary to induce cellular responses, in accordance with ELISPOT results (see 5.5.4). The IFN-γ production was less evident in $CD8\text{-}\beta^+$ T-cells, being the highest response in B_2T-3D3A group (1.7 ± 0.1 %), following B_2T-3D (1 ± 0.1 %), B_2T-3A3D (0.9 ± 0.1), B_2 [pig 98 (0.8 %)] and B_2T-3A (0.7 ± 0.4 %). These results indicated that cytotoxic $CD8\beta^+$ T-cells are less frequently stimulated by the dendrimers comparing $CD4^+$ population (**Figure 24B**).

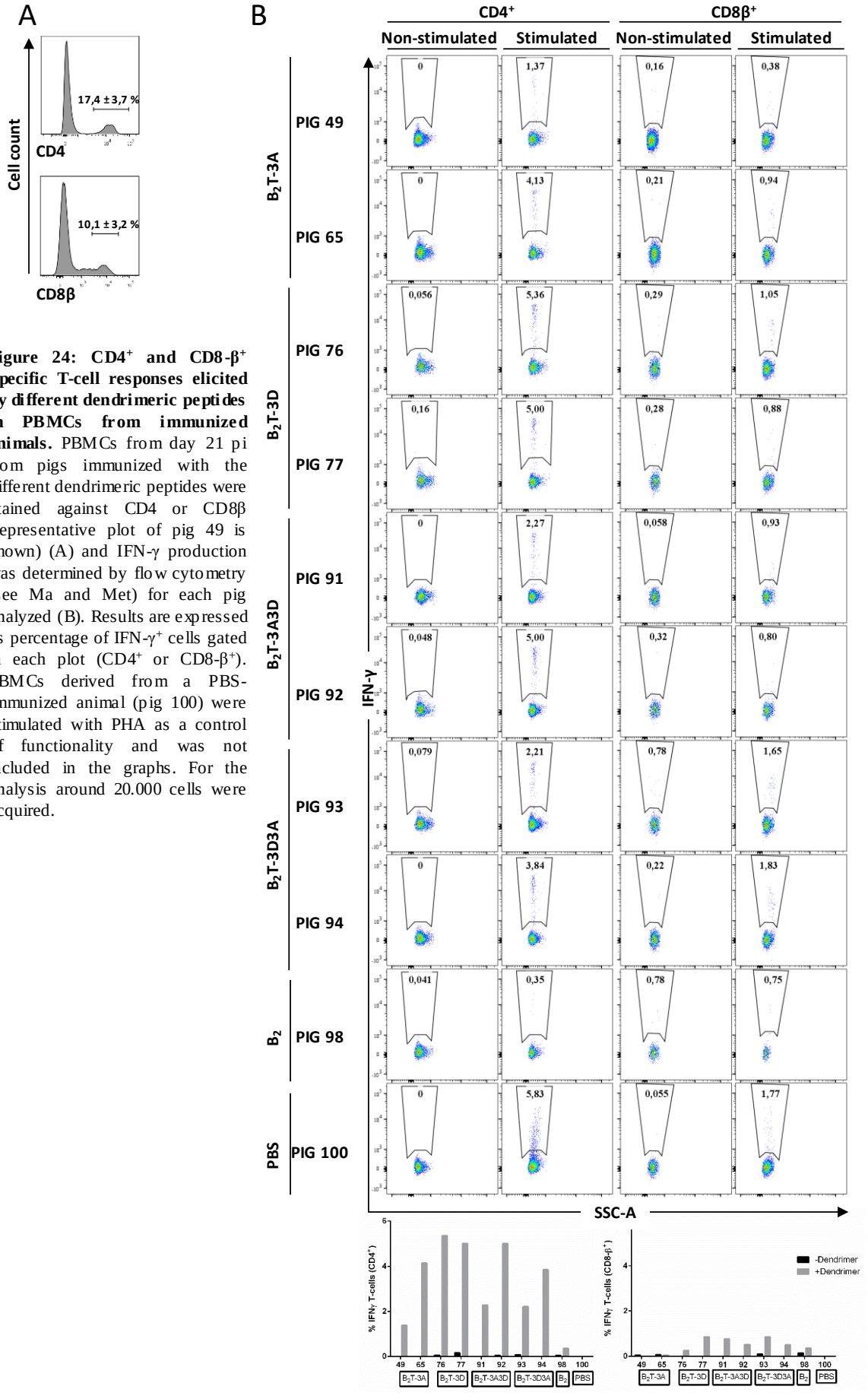


Figure 24: CD4$^+$ and CD8-β$^+$ Specific T-cell responses elicited by different dendrimeric peptides in PBMCs from immunized animals. PBMCs from day 21 pi from pigs immunized with the different dendrimeric peptides were stained against CD4 or CD8β (representative plot of pig 49 is shown) (A) and IFN-γ production was determined by flow cytometry (see Ma and Met) for each pig analyzed (B). Results are expressed as percentage of IFN-γ$^+$ cells gated in each plot (CD4$^+$ or CD8-β$^+$). PBMCs derived from a PBS-immunized animal (pig 100) were stimulated with PHA as a control of functionality and was not included in the graphs. For the analysis around 20.000 cells were acquired.

5.6.2 Depletion of $CD4^+$ T-cells from PBMCs abolishes IFN-γ production

To confirm the phenotype of the T-cell subset responsible for IFN-γ expression, $CD4^+$ T-cells were depleted from PBMCs isolated from a B_2T-3D immunized pig that resulted a good responder (pig 87) using a specific mAb and magnetic beads in a MACS column. The positive fraction magnetically isolated ($CD4^+$) enriched in $CD4^+$ T-cells, and the negative fraction ($CD4^-$) depleted of $CD4^+$ T-cells, were *in vitro* stimulated with B_2T-3D or peptide T-3D and the expression of IFN-γ was determined by ELISPOT. Total PBMCs and the reconstitution of both fractions ($CD4^+$/$CD4^-$) were included as controls. Flow cytometry of each fraction was performed to confirm the efficacy of the purification. As shown in **Figure 25A**, 21.8 % of initial PBMCs were $CD4^+$. After the sorting, the eluted negative fraction ($CD4^-$) showed a clear reduction of $CD4^+$ cells (3.4 %) and the positive fraction ($CD4^+$) showed a notable increment in $CD4^+$ cells (97.7 %) proving that the fractions sorted, was highly purified for CD4 marker (**Figure 25A**). No fluorescence was detected in non-stained cells (data not shown). Next, initial PBMCs and each magnetically sorted fraction was stimulated with B_2T-3D and T-3D peptides and IFN-γ expression was measured by ELISPOT. As shown in **Figure 25B** the fraction enriched in $CD4^+$ T-cells elicited the highest levels of IFN-γ secreting cells upon both, dendrimer and T-cell epitope stimulation. On the other hand, $CD4^-$ population did not produce IFN-γ spots. As expected, total PBMCs and the reconstituted fraction $CD4^+$/$CD4^-$ showed similar levels of IFN-γ secreting cells.

This result suggests that $CD4^+$ T-cells are the main population responsible for specific IFN-γ release and the major subset involved in T-cell immunity evoked by dendrimers.

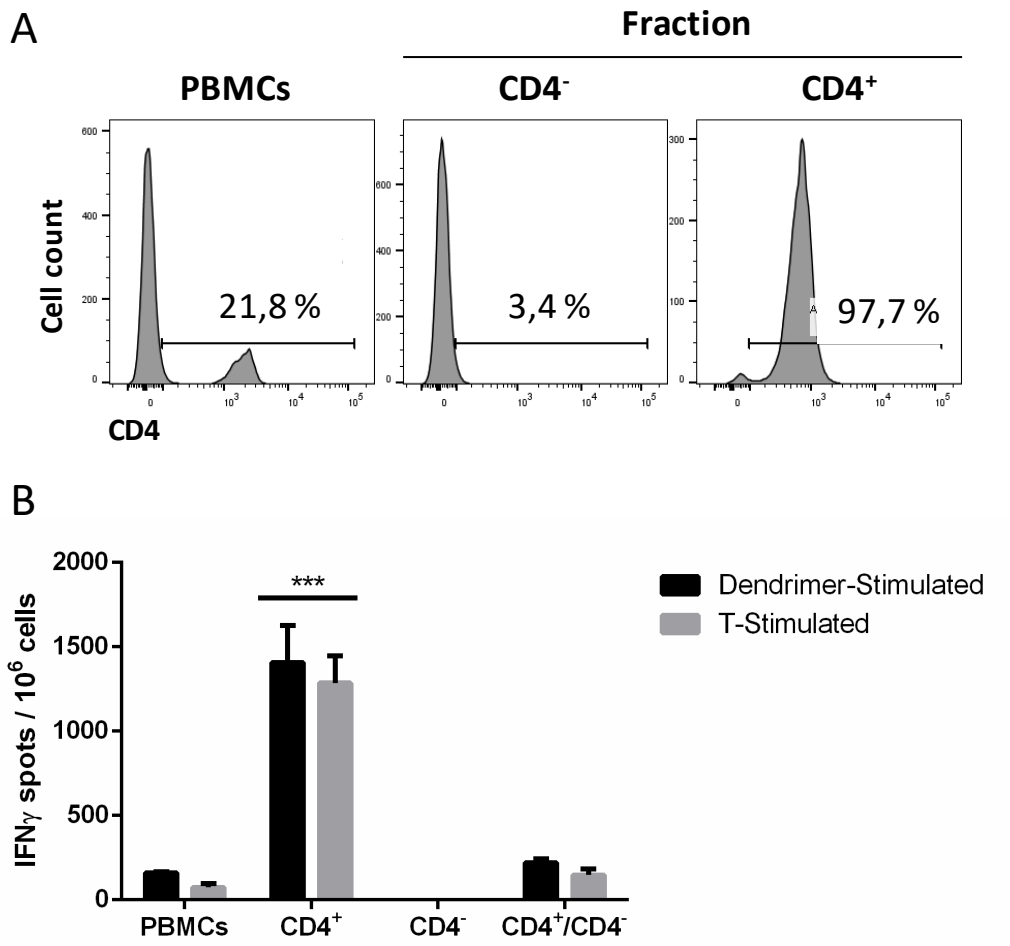


Figure 25: Depletion of CD4$^+$ T-cells abolishes IFN-γ production of peptide-stimulated PBMCs. Cells from a B_2T-3D immunized pig (pig 87) were magnetically sorted using anti-CD4 mAb. A) FACS analysis was performed to determine the CD4 staining quality of initial pre-sorted PBMCs and each purified fraction after MACS fractioning. B) Pre-sorted PBMCs and fractioned positive fraction (CD4$^+$), negative fraction (CD4$^-$) and reconstituted fraction (CD4$^+$/CD4$^-$) were plated in triplicates and stimulated either with B_2T-3D or T-3D peptides for 48 h and IFN-γ production was determined by ELISPOT. The values are presented as IFN-γ spots per 10^6 cells (spots from mock-stimulated cells are substrated). Each column represents the mean of a triplicate ± SD. Statiscally significant differences are indicated by three asterisks (***) for $p < 0.001$.

5.6.3 Memory CD4$^+$ T-cells are the responsible subset of the IFN-γ secretion

To evaluate the CD4$^+$ T-cell subset responsible for the IFN-γ production induced by the dendrimers, PBMCs from groups of two pigs immunized with each of the B_2T-immunized groups (as in 5.6.1), were stimulated with the homologous dendrimer and stained with an antibody recognizing the selective surface marker 2E3. This marker has been described to be expressed on the surface of naïve CD4$^+$ cells in swine PBMCs but not in memory CD4$^+$ cells (Revilla et al., 2004). After staining, cells were permeabilized, IFN-γ was detected using a specific mAb and a flow cytometry analysis was performed to quantify positive IFN-γ cells (see Materials and Methods 4.14.2). Next, CD4$^+$ lymphocytes were gated and 2E3 expression was analyzed in this subset.

The percentage of $CD4^+2E3^+$ and $CD4^+2E3^-$ cells in the pigs varied, being higher for the latter subset (3.3 ± 1.3 % and 8.9 ± 3.2 % respectively) (**Figure 26A**). $CD4^+2E3^+$ subset barely produced IFN-γ, being the levels higher in pigs immunized with B_2T-3A (1.5 ± 0.6 %), followed by B_2T-3A3D (0.9 ± 0.6 %), B_2T-3D (0.4 ± 0.3 %) and B_2T-3D3A and B_2 (0.2 ± 0.2 %) groups.

Interestingly, a clear induction of IFN-γ was prominent in the $CD4^+2E3^-$ cells in all immunized groups upon homologous peptide stimulation. Thus, $CD4^+2E3^-$IFN-γ^+ subsets were more prominent in B_2T-3D (4.6 ± 0.8 %) followed by B_2T-3A3D (4.2 ± 1.4 %), B_2T-3A (3.6 ± 0.5 %) and to lesser extent B_2T-3D3A (1.2 ± 0 %) and B_2 (1.3 %) groups (**Figure 26B**). Noteworthy, animals immunized with B_2T-3D3A (pigs 93 and 94) and B_2 (pig 98) showed, in average, less IFN-γ production than those of the rest of the groups (**Figure 26B**). No fluorescence signal was detected in unstimulated cells (data not shown).

These results suggest that memory $CD4^+$ T-cells are the major subset involved in IFN-γ production and in the T-cell immunity elicited by B_2T dendrimers.

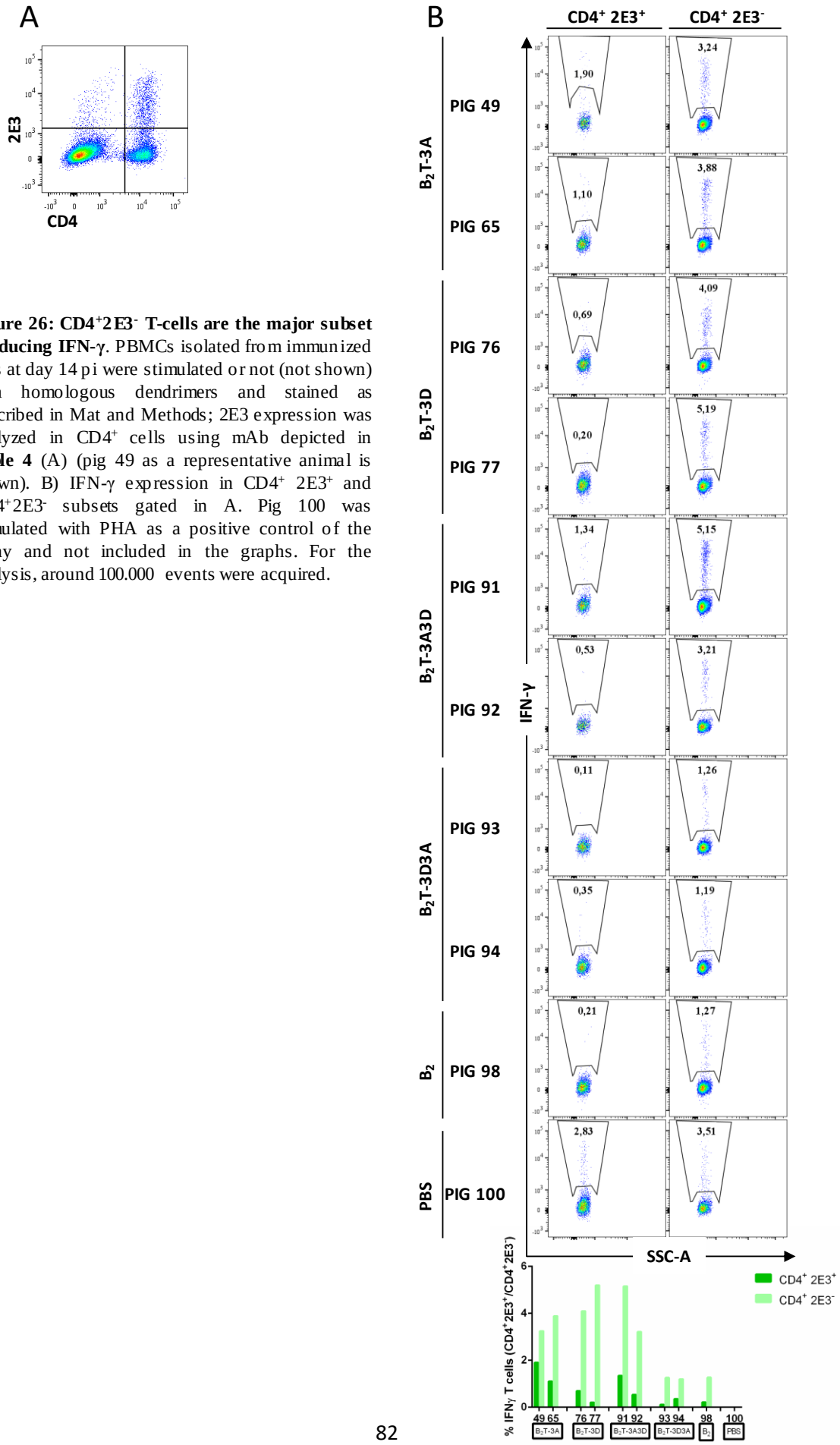


Figure 26: CD4⁺2E3⁻ T-cells are the major subset producing IFN-γ. PBMCs isolated from immunized pigs at day 14 pi were stimulated or not (not shown) with homologous dendrimers and stained as described in Mat and Methods; 2E3 expression was analyzed in CD4⁺ cells using mAb depicted in **Table 4** (A) (pig 49 as a representative animal is shown). B) IFN-γ expression in CD4⁺ 2E3⁺ and CD4⁺2E3⁻ subsets gated in A. Pig 100 was stimulated with PHA as a positive control of the assay and not included in the graphs. For the analysis, around 100.000 events were acquired.

5.7 Multifunctional $CD4^+$ T-cells are activated by the B_2T dendrimers

Studies on multifunctional T-cells, *i.e*. T-cells performing several functions like cytokine production or degranulation, have revealed that these cells play a central role in protective immune responses (Sallusto and Lanzavecchia, 2009). As commented in the Introduction (see section 2.4), IFN-γ is the main effector cytokine expressed in Th1 T-cells, but other pro-immflamatory cytokines are also involved in Th1 effector responses and are important in T-cell immunity. In order to evaluate whether $CD4^+$ T-cells activated by the B_2T dendrimers were producing other effector cytokines apart from IFN-γ, the expression of IFN-γ along with that of TNF-α (double positives) was analyzed by flow cytometry. To this end, an approach similar to that in 5.6 was followed. Thus, PBMCs stimulated or not with homologous dendrimers, were stained with surface markers and their intracellular IFN-γ and TNF-α labelled.

$CD4^+$ cells were clearly detected with percentages that were variable among the pigs analyzed (11.1 ± 1.9 %) (**Figure 27A**; (representative plot of pig 91 is presented). When the expression of IFN-γ and TNF-α was analyzed in $CD4^+$ T-cells, a strong fluorescence signal was detected in PBMCs stimulated with homologous peptides (**Figure 27B**).

In general, percentages of single cytokine-expressing cells were low for both cytokines, $CD4^+$ IFN-γ^+ TNF-α^- (B_2T-3A: 0.9 ± 0.4 %; B_2T-3D: 0.5 ± 0.1 %; B_2T-3A3D: 0.3 ± 0.2 %; B_2T-3D3A: 0.2 ± 0.1 % and B_2 0.2 %) and $CD4^+$ IFN-γ^- TNF-α^+ (B_2T-3A: 1.4 ± 0.1 %; B_2T-3D: 0.8 ± 0.5 %; B_2T-3A3D: 0.5 ± 0.1 %; B_2T-3D3A: 0.3 ± 0.1 % and B_2: 0.7 %). Most of the activated $CD4^+$ T-cells expressed both cytokines, which is consistent with multifunctional $CD4^+$IFN-γ^+TNF-α^+ phenotype. This was particularly evident in pigs immunized with B_2T-3A (2.7 ± 1.9 %), B_2T-3D (2 ± 0.6 %) and B_2T-3A3D (1.5 ± 0.8 %). In contrast, in B_2T-3D3A (0.8 ± 0.1 %) and B_2 [pig 98 (0.5 %)] groups lower levels of cells expressing both cytokines were observed (**Figure 27B**).

These results are consistent with data presented in previous sections (5.6.1 and 5.6.3), and suggest again that not only the presence, but also the orientation of T-cell epitopes influences the T-cell response evoked by the dendrimer peptides.

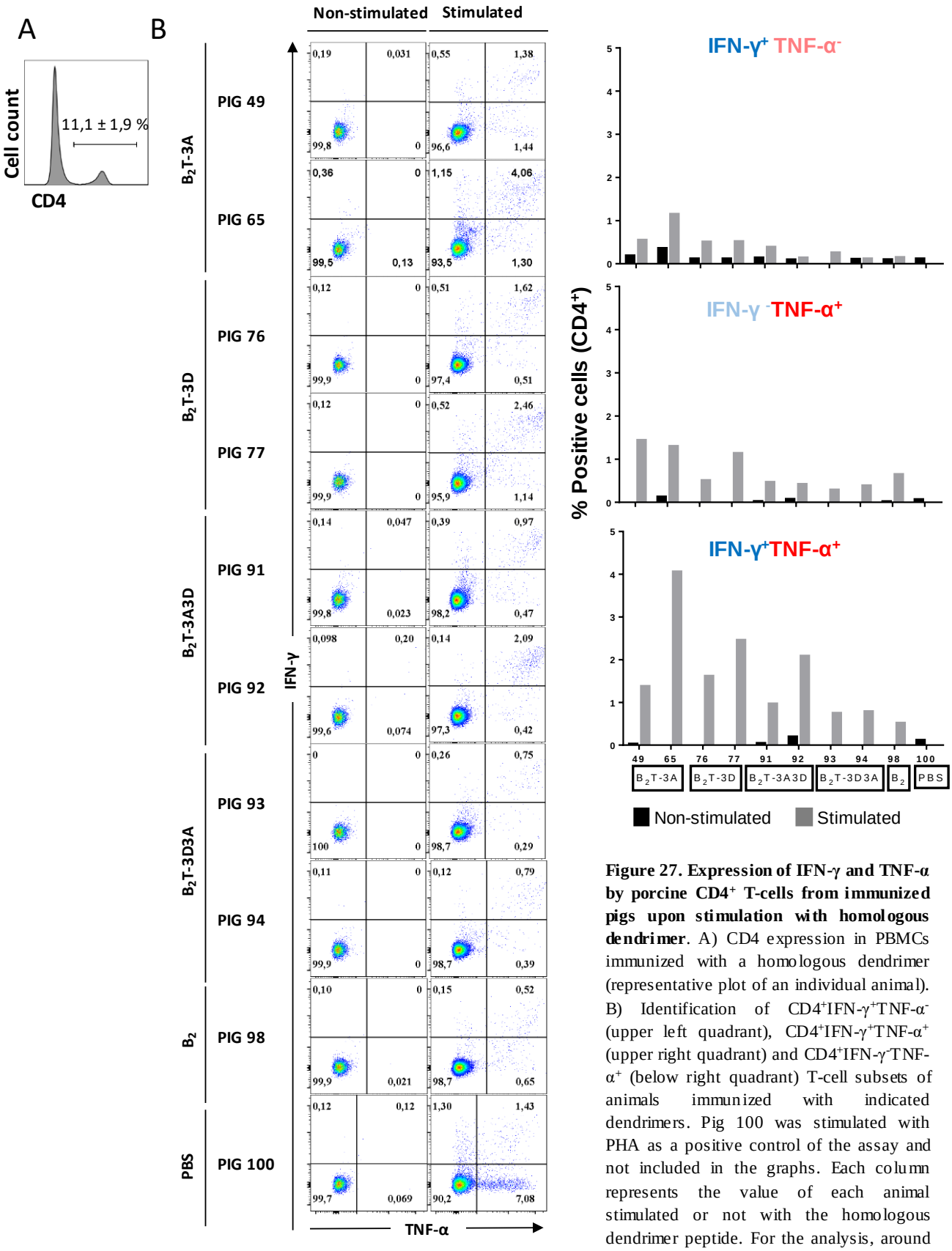


Figure 27. Expression of IFN-γ and TNF-α by porcine CD4⁺ T-cells from immunized pigs upon stimulation with homologous dendrimer. A) CD4 expression in PBMCs immunized with a homologous dendrimer (representative plot of an individual animal). B) Identification of $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^-$ (upper left quadrant), $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ (upper right quadrant) and $CD4^+IFN\text{-}\gamma^-TNF\text{-}\alpha^+$ (below right quadrant) T-cell subsets of animals immunized with indicated dendrimers. Pig 100 was stimulated with PHA as a positive control of the assay and not included in the graphs. Each column represents the value of each animal stimulated or not with the homologous dendrimer peptide. For the analysis, around 20.000 cells were acquired.

5.8 The dendrimer peptides can induce nAbs against a broad spectrum of type O FMDVs

As commented in the Introduction (see 2.2 and 2.5.1), the highly antigenic diversity of FMDV reflected in 7 different serotypes with multiple variants each, makes the development of vaccines a challenging issue. Since type O FMDVs are responsible of most of the current FMD outbreaks in endemic countries, a broad-spectrum response is necessary for optimal vaccines against this serotype. Therefore, we were interested in assessing the neutralization range afforded in pig by the B_2T dendrimers studied. To this end, sera recovered from the three different experiments in which pigs were vaccinated with dendrimer peptides were tested for its capability to neutralize a panel of type O FMDVs (**Figure 28**). The FMDV isolates selected belonged to different type O topotypes, *i.e* viruses from different spatiotemporal locations (see Materials and Methods 4.2). A different non-related serotype C FMDV isolate (CS8-c1) was included as a serotype-specific control.

All dendrimer constructions, but B_2 as expected, were able to induce nAbs against the panel of FMDVs showing similar nAb titers. Constructions B_2T-3A as well as B_2T-TB_2 with a high or a low dose, induced nAbs against all the FMDVs tested without significant differences among the viruses compared (**Figure 28A**). This effect was also observed with B_2T-3D (**Figure 28B**), B_2T-3A3D and B_2T-3D3A immunized sera, although to a lesser extent in the later (**Figure 28C**). As expected, no nAb were detected in B_2-immunized animals, confirming our previous results with homologous O/UK/11/2001 virus (see 5.5.2). The level of neutralization afforded by the different dendrimers against the battery of viruses relative to the homologous isolate O/UK/11/2001 (r_1 value) is shown in **Figure 28D**. The r_1 value is the ratio of VNT between the virus problem/the homologous virus (see 4.11). All of the viruses tested showed similar or even higher r_1 values than that of O/UK/11/2001. Interestingly, some viruses such as O_1Campos and O_1BFS were neutralized to a higher extent than the homologous virus O/UK/11/2001. As expected, the neuralizations observed were serotype-specific and none of the sera from any immunized groups was able to neutralize type C CS8-c1 virus (**Figure 28D**).

These results support that B_2T dendrimers induce a broad anti-FMDV immunity within a serotype, which can be considered as an important valuable asset for their potential use in endemic countries where a wide spectrum of antigenic variable pools of FMDVs can circulate.

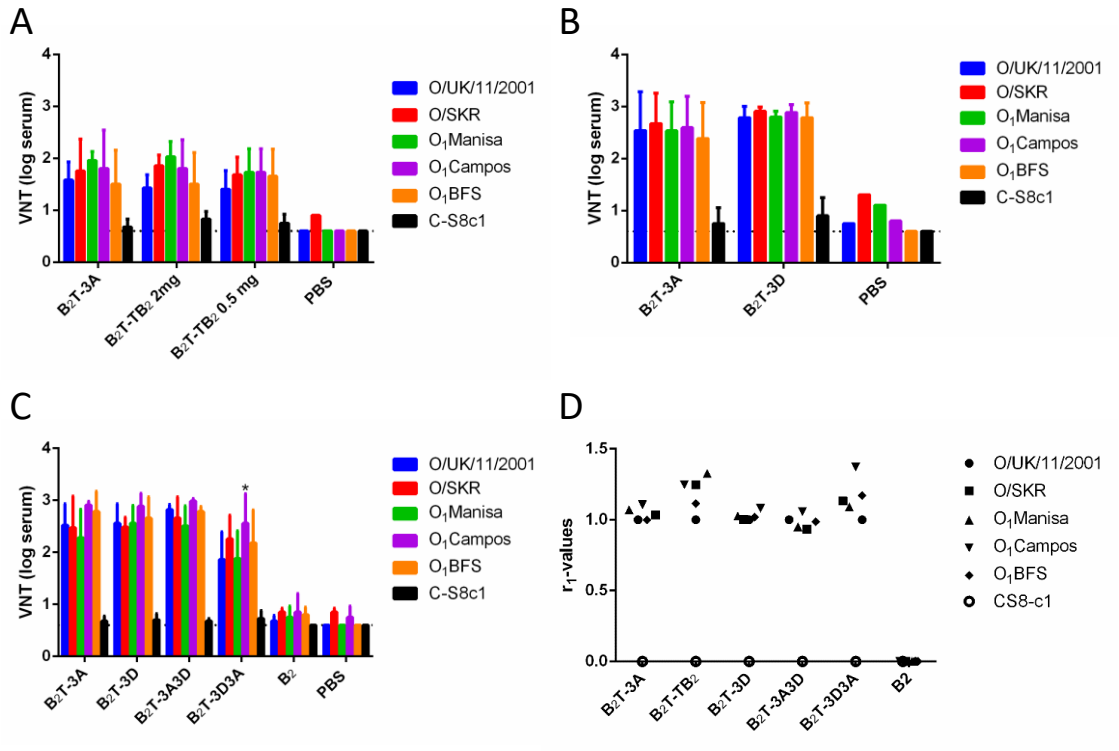


Figure 28: Sera from pigs immunized with B_2T dendrimers can neutralize a wide panel of different FMDVs type O topotypes. Sera recovered from animals immunized with the different peptides used in the first experiment (A), the second experiment (B) and the third experiment (C) were tested to neutralize a panel of different type O FMDVs. D) Antigenic relationship (r_1) values of the six viruses. The serological match (r_1-values) of the different peptides is shown as symbols and is calculated as described in Materials and Methods (section 4.11). Individual columns represent the mean of each group (n=4) ± SD. Values are expressed as the reciprocal $\log_{10}$ of the last serum dilution that neutralized 100 TCID50 of each FMDV. Statiscally significant differences are indicated by one asterics (*) for $p < 0.05$.

5.9 Towards a FMDV universal vaccine: immunogenicity in mice of non-covalent mixtures of B_2T-3As peptides from different serotypes

Polyvalent conventional vaccines incorporate inactivated viruses from different FMDV serotypes, which should match the potential circulating viruses according to the epidemiological status of each region/country. To explore the capability of our dendrimer platform to allow heterotypic immunization, we assessed the nAbs elicited by a mixture of two independent dendrimer peptides (B_2T-3A) differing in the B-cell epitope. To this end, groups of eight mice were immunized with: i) a non-covalent equimolar mixture of B_2T-3A containing the B-cell epitope from the type O FMDV O/UK/11/2001, here termed B_2T-3A-O, and its version enclosing the B-cell epitope from the type C CS8-c1 isolate, termed B_2T-3A-C, ii) B_2T-3A from type C (B_2T-3A-C), or iii) B_2T-3A from type O (B_2T-3A-O) (**Figure 29**).

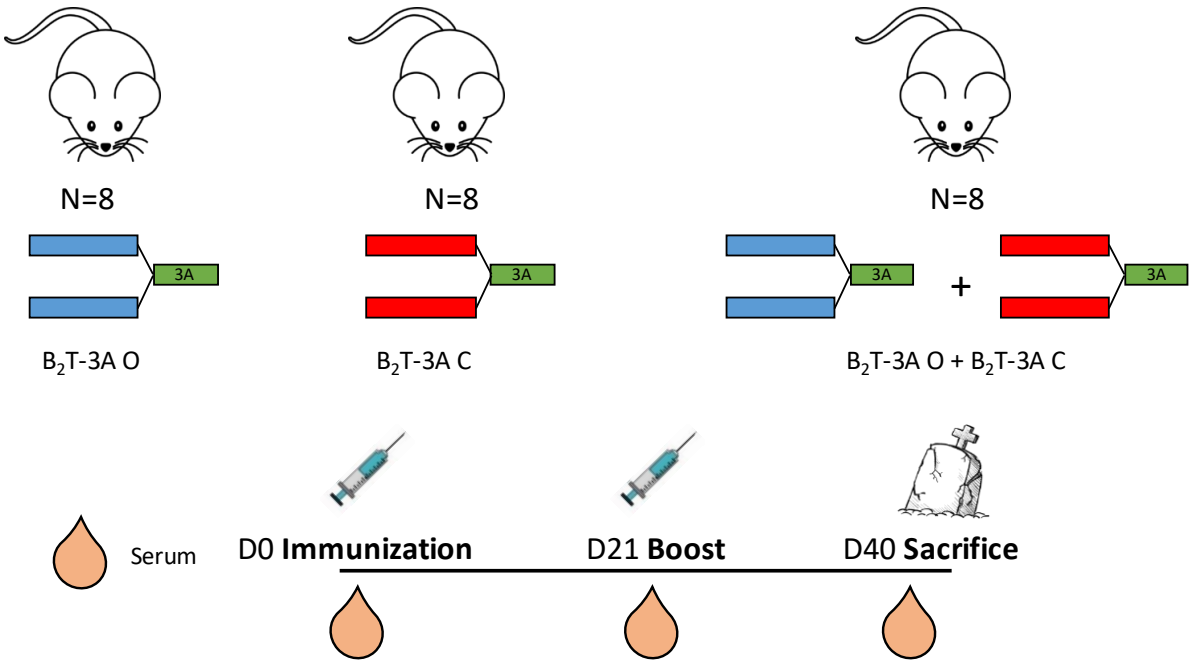


Figure 29: Experimental scheme of the immunization of mice with mixtures of heterotypic B_2T-3A peptides. Groups of eight mice were immunized with 100 µg of either B_2T-3A-O (type O) or B_2T-3A-C (type C) or with the mixture of 100 µg of each of the peptides in a single shot (B_2T-mixture). Animals were boosted at day 21 and euthanized at day 40. Sera samples were collected at the days indicated.

5.9.1 Combination of B_2T-3A peptides harboring heterotypic B-cell peptides elicits similar titers of type-specific FMDV nAb in mice

Homologous neutralization assays were used to determine the capacity of sera from mice immunized twice with B_2T-3A-C or B_2T-3A-O to neutralize C-S8c1 or O/UK/11/2001 virus, respectively. In parallel, the neutralization of these two viruses by sera from the B_2T-mix group was analyzed. In this experiment, no nAbs were observed at day 21 pi (data not shown).

When the capacity to neutralize type O FMDV was observed (day 40 pi), similar nAb titers were noticed in both, in B_2T-3A-O and in B_2T-3A mixture-immunized mice (2.1 ± 0.8 *vs* 2.2 ± 0.8 $\log_{10}$) (**Figure 30A**). Likewise, animals in the B_2T-3A-C group, reached VNT to type C CS8-c1 similar as those observed in the B_2T-3A mixture group (2.2 ± 0.8 *vs* 2.4 ± 0.8 $\log_{10}$) (**Figure 30B**). When the neutralizing activity elicited against the two viruses was compared, six out of eight animals (75 %) showed high and similar titers, although the correlation was not absolute (**Figure 30C**).

Thus, these data suggest that non-covalent mixtures of B_2T-3As can induce potent anti-FMDV antibodies against viruses from two different serotypes, open the possibility of

developing a feasible vaccine platform against a broad spectrum of serologically different FMDVs.

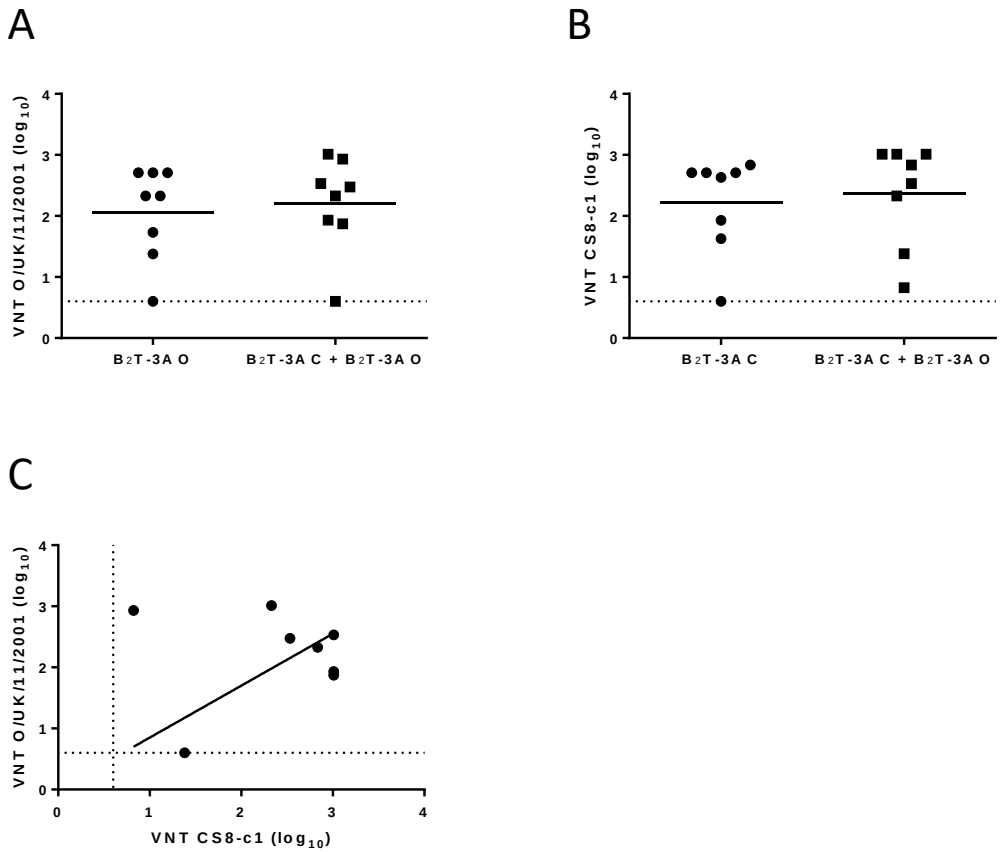


Figure 30: **The mixture of two heterotypic B_2T-3As elicits similar levels of nAb against type C and type O FMDVs**. nAb titers in sera samples from B_2T-3A mixture-immunized mice at day 40 pi against type O FMDV (A) and type C FMDV (B). (C) Lineal regression analysis comparing the neutralizing activity form B_2T-mixture against both viruses. Each point represents the mean of a triplicate of each animal (n=8). Dotted lines indicate the detection limit of the assay. Values are expressed as the reciprocal $\log_{10}$ of the last serum dilution that neutralized 100 TCID50 of each FMDV.

5.10 Dissecting the immunogenicity of B_2T-3A: constructions lacking integrin binding motif in the B-cell epitope

The B-cell epitope included in the B_2T dendrimer constructs studied in this Thesis, contains the three amino acid motif RGD that plays a critical role as the virus ligand to cellular integrins, such as $\alpha_v\beta_6$, known to act as the main FMDV cell receptors *in vivo* (see Introduction, section 2.2). As integrins are expressed in the surface of DCs as well as in a variety of other cell types (Hynes, 1987), it can be speculated that abolishing the interaction of B_2T peptides with integrin receptors could modulate dendrimer uptake by APCs and its recognition by immune cells and, therefore, the capacity to elicit protective immune responses. To this end, two B_2T-3A versions in which the B-cell epitope was

that of CS8-c1 FMDV harboring amino acid substitutions previously reported to inhibit the binding to integrins but maintaining the reactivity against mAbs to CS8-c1 FMDV (Verdaguer et al., 1998) were syntesized: B_2T (R141H in VP1) and B_2T (R141K in VP1). First, the binding of these variant dendrimers to integrins was assessed *in vitro* by determining its capacity to prevent virus attachment and infection in cultured cells. Thus, a range of concentrations of 25, 2.5 and 0.25 µM of each peptide were incubated in confluent BHK-21 cell monolayers and the inhibition of infection was determined by plaque assay. As shown in **Figure 32A**, the two variant dendrimers did not affect the infection yield, while B_2T and B peptides impaired infection in the range of peptide concentration tested. An irrelevant peptide T-3A used as a negative control, exerted a 50% inhibition at 25 µM concentration.

To test whether these dendrimers, with an impaired integrin binding, could modulate the immune response of B_2T-3A, groups of ten mice were immunized with the canonical B_2T, or with peptides B_2T-R141H or B_2T-R141K, and the neutralizing ability of the sera was analyzed. A detail diagram is presented in **Figure 31**.

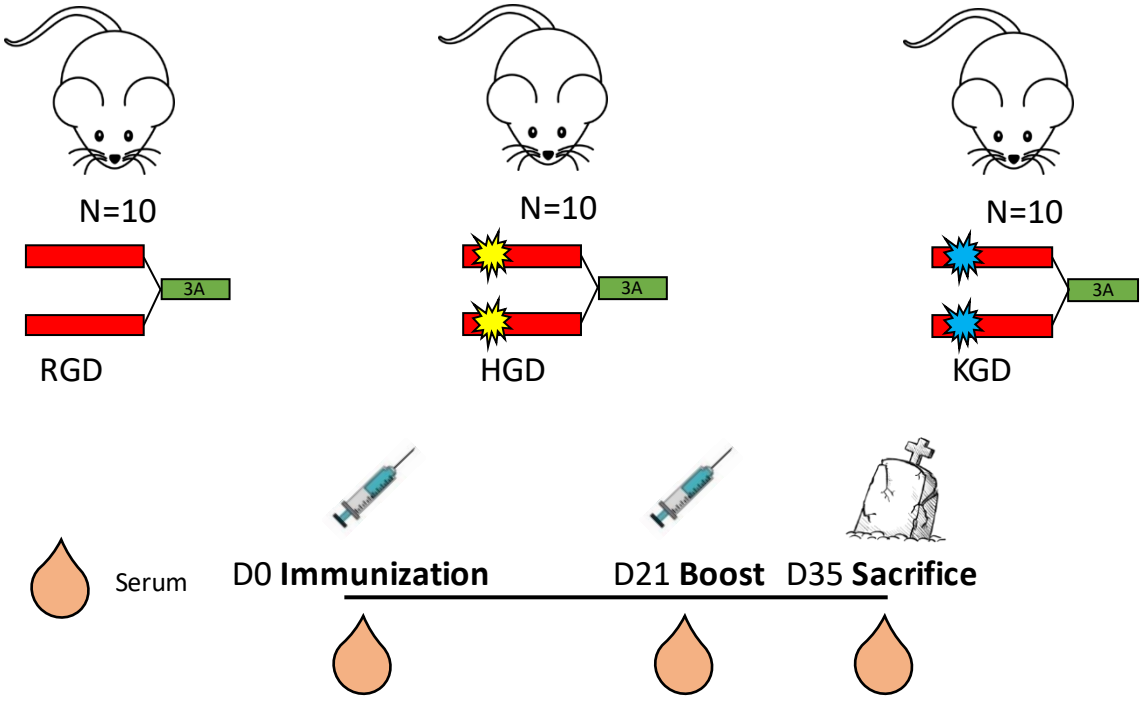


Figure 31: Experimental scheme followed to analyzed the neutralizing activity of B_2T dendrimers with an impaired integrin-binding site. Briefly, groups of ten mice (n=10) were immunized subcutaneously with 100 ug of each dendrimer at day 0 and boosted at day 21 pi. Sera samples were collected at the indicated days and neutralizing activity against the homologous virus (CS8-c1) was analyzed as previously.

After one peptide dose, at day 21 pi, all the mice showed detectable levels of nAbs in the B_2T group (2.4 ± 1 log_{10}) and a reduction of titers was observed in animals immunized with each of the variant peptides [B_2T (R141H): 1.6 ± 0.6 log_{10}; B_2T (R141K): 2.2 ± 0.5 log_{10}] (**Figure 32B**). After the second peptide dose, at day 35 pi (14 post-boost), a notable increment in the titers was observed in the three groups [B_2T: 3.1 ± 0.3 log_{10}; B_2T (R141H): 2.3 ± 0.8 log_{10}; B_2T (R141K): 2.5 ± 0.5 log_{10}]. In this case, the titer reductions observed with the variant dendrimers were statistically significant in animals with B_2T (R141H) (**Figure 32C**).

These results suggest that the lack of the RGD motif in the B_2T vaccines, which prevents its attachment to integrins, lead to the induction of FMDV nAb although to a lesser extent than the original B_2T construction, not improving the immunogenicity of the dendrimer.

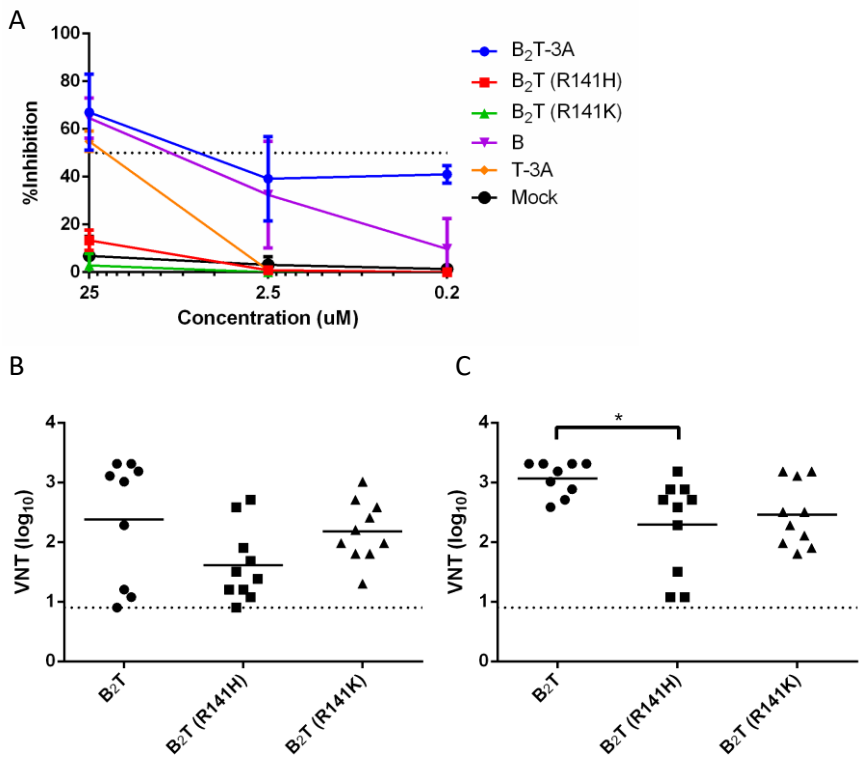
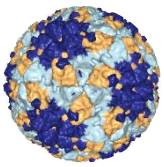


Figure 32: B_2T dendrimers unable to bind integrin induce lower nAb titers against homologous virus. A) B_2T peptides harboring single amino acid substitution on the B-cell epitope, previously reported to inhibit binding to integrin were tested for its ability to prevent CS8-c1 infection in BHK-21 cells. The dotted line indicates the concentration at which a reduction of 50% of plaque formation is observed (IC_{50}). Groups of ten mice (n=10) were immunized twice with B_2T or The B-cell epitope-modified versions B_2T R141H or B2T R141K and nAbs titers were analyzed after one dose at day 21 pi (B) or after a second dose at day 35 pi (14 post-boost) (C). Each point represents the mean of a triplicate of each animal (n=10). Dotted lines indicate the detection limit of the assay. Values are expressed as the reciprocal log_{10} of the last serum dilution that neutralized 100 TCID50 of CS8-c1 FMDV. Statically significant differences are denoted as * for $p<0.05$ using two-way ANOVA and Tukey's post-hoc correction.



DISCUSSION

6. Discussion

The use of peptides as valuable vaccines against viral diseases has gained interest in the last years (Heegaard et al., 2010; Li et al., 2014; Rosendahl Huber et al., 2014). This expectative also applies to FMDV, since current commercial vaccines have serious handicaps (see Introduction 2.5.1). Indeed, the search of alternative vaccines, including those against FMD, has come to the fore of the animal health (Blanco et al., 2017; de Los Santos et al., 2018). In our group, we are interested in developing next-generation FMD vaccines, in particular those based on synthetic peptides. Among their advantages, highlight their safety (no handling of live virus is needed), thermal stability, easy production and manufacturing, and clear differentiation between infected and vaccinated individuals, since peptides lack antigenic sites from NSPs that could produce infection-specific antibodies.

The potential of linear peptides to confer protection against FMD was reported in the early 80's (Bittle et al., 1982; DiMarchi et al., 1986; Kleid et al., 1981). However, the limited protection that linear peptides conferred in natural hosts has hampered their final consideration as feasible vaccines (Cubillos et al., 2012; Taboga et al., 1997). To overcame this limitation, our group is working in assessing the immunogenic potential of multiple antigenic peptides, termed dendrimers, in which branched synthetic peptides harboring different B and T-cell epitopes are displayed in single molecules. In line with previous data (Blanco et al., 2013; Blanco et al., 2016; Cubillos et al., 2008), the results presented here contribute to the understanding of the immune mechanisms behind the B and T-cell responses elicited by FMDV dendrimers and confirm the potential of these molecules as serious candidates for FMD vaccines. Indeed, dendrimer platforms are among the multimeric strategies being currently considered as promising alternatives for vaccine development against diseases for which vaccines are not still available, such as malaria, hepatitis C virus (HCV), human immunidefiency virus (HIV) or diverse types of cancer (Kunwar et al., 2013; Lamonaca et al., 1999; Lennerz et al., 2014; Tsuji and Zavala, 2001).

6.1 Immunogenicity of dendrimer peptides in the mouse model

The use of laboratory animal models to study human or veterinary diseases remains as a current demand. Regarding vaccine design, the use of surrogates species can introduce differences relative to target species, as those derived from differences in MHC class I

and II allele composition (Koff et al., 2013; Li et al., 2014; Rosendahl Huber et al., 2014). Thus, although the results in animal models have to be considered cautiously, they have become irreplaceable tools for translational medicine. FMDV research is not an exception and mice have been widely used to study pathogenesis, antivirals and immune response against vaccine candidates (Habiela et al., 2014). Moreover, the mouse model is of special interest because of its low cost, relative low housing requirements, wide availability of reagents and knockout technology, allowing detailed studies that cannot be addressed in natural FMD hosts.

Mice susceptibility to FMDV infection depends on the mouse strain and the viral isolate considered. However, it is well documented that mice induce nAbs upon FMDV infection or vaccination (Borca et al., 1986; Perez Filgueira et al., 1995; Salguero et al., 2005). Our group previously reported that alternative subunit vaccines consisting of multiple FMDV antigenic peptides induced similar nAb titers in outbred Swiss mice as those elicited in a natural host: the pig (Blanco et al., 2013). Hereby, in this Thesis, a panel of variants of dendrimer B_2T-3A either harboring different porcine T-cell epitopes or constructed using different chemistries, were analyzed in mice as a screening step to select those more promising to be tested in the pig (**Figure 7**). Although most dendrimers induced specific Abs detected by ELISA that neutralized the homologous virus *in vitro*, the results obtained with these peptides in mice have to be interpreted rigorously since the immune response elicited in mice and in FMDV natural hosts can be dramatically different (Nunez et al., 2007; Rodriguez et al., 2003). Besides the differences in the MHC restriction commented above, the route of vaccine delivery in mice may differ from that in vaccinated natural hosts and the antigen can interact with different immune cell populations. A clear example of that is shown in this Thesis, in which peptide B_2T-3D, which did not induce Ab in mice, turned to be a good immunogen in pigs, highlighting the differences between both species. Conversely, the results in mice of the tail-to-tail joined version of B_2T-3A, the B_2T-TB_2 dendrimer, paralleled those observed in pigs. Thus, our results confirm the limitation to extend the results on the the immunogenicity of dendrimer peptides in mice to FMDV natural hosts (Cunliffe and Blackwell, 1977; McVicar et al., 1973).

Regarding the humoral immune response elicited by the different peptides, dendrimers B_2T-3A and B_4T induced specific anti-FMDV nAbs in mice, as previously reported (Blanco et al 2013). Interestingly, B_2T-TB_2 induced higher nAb titers, confirming that

dimerization of B_2T-3A has a positive effect on immunogenicity. Probably the key of the immunogenicity of B_2T-TB_2 platform relies on the juxtaposed presentation of the B-cell epitopes, reducing steric interactions among the four copies of the B-cell peptide. This is in contrast to B_4T (tetravalent) that elicited, an antibody response lower than that of B_2T-3A, which could be due to a higher local density of the B-cell peptide that would reduce the cross-linking efficacy with the specific B-cell receptor (paratope). Conversely, B_2T-3D did not induce detectable nAb in any of five experiments performed. Pioneering work performed by Francis et al., 1987 demonstrated that antibody response against the B-cell epitope located in the VP1 G-H loop is T-cell dependent. More recent studies have confirmed this in cattle infected with FMDV or immunized with inactivated virus (Carr BV et al.,2013; Juleff et al.,2009). Our results suggest that the 3D epitope presented in the B_2T platform is not efficiently recognized as an efficient T-helper epitope in mice. Thus, 3D epitope sequence probably has not high affinity for mouse MHC haplotypes. Nevertheless, we cannot rule out that an inefficient processing of the epitope and/or conformation alterations affecting to the correct cross-linking of the B-epitope can also be contributing to the lack of antibody induction observed. Further work is required to experimentally confirm the lack of recognition of T-3D and to a lesser extent of T-3C, which were identified as T helper epitopes in swine, by murine T-cells. In any case, our results evidence the limitations of the mouse model for the analysis of the role of FMDV-specific T-cell epitopes in the protective responses in natural host species, such as the pig.

6.2 Immunogenicity of dendrimer peptides in swine

Swine was the FMDV host species used for assessing the immunogenicity of the dendrimer peptides selected from the results obtained in the mouse experiments. This choice was due to the possibility of performing experiments with pigs in high containment facilities at the BSL-3 FMDV laboratory, CISA-INIA (Valdeolmos, Spain). Moreover, the importance of the pig as FMDV target species in animal health is increasing, as the main incidence of the disease shifts towards Asian countries (Perez and Willeberg, 2017).

In general, animal-to-animal variation is a common feature in the experiments performed, as observed in previous studies with peptide and other subunit vaccines (Blanco et al., 2016; Cubillos et al., 2008; Gullberg et al., 2016; Rodriguez et al., 2003; Soria et al., 2017; Taboga et al., 1997; Wang et al., 2002). As in all FMDV natural hosts, the genetic background of individual pigs may differ from each other. Thus, a pool of MHC (SLA in swine) alleles exists in the population and this polymorphism can contribute to the

individual variability observed. In this line, vaccine research has to deal with individual variations since some individuals may respond better or worse to vaccines than others (Koff et al., 2013; Li et al., 2014). This is more marked in subunit vaccines such as our B_2T dendrimers, in which the limited T-cell epitopes included are SLA-restricted (Collen et al., 1991; Garcia-Briones et al., 2000; Taboga et al., 1997). Thus, the inclusion of epitopes recognized by a wide range of MHC molecules is necessary for vaccine effectiveness (Li et al., 2014; Rosendahl Huber et al., 2014). The T-cell epitopes incorporated in our dendrimers were previously described as heterotypic (conserved among different FMDV serotypes) and promiscuous (widely recognized by domestic pig individuals) (Blanco et al., 2001).

In a first attempt to correlate the immune response elicited by the dendrimers and the SLA haplotypes expressed in the pigs, in collaboration with Dr. Sabine Hammer (Vienna University), genetic haplotyping of all the animals tested so far (from experiments presented in this Thesis and in a retrospective way with previous, related experiments), is currently ongoing. This data will be used to elucidate which SLA allele/s are associated with higher/lower immune responses and, eventually, in protection/non-protection. Preliminary data suggest that certain SLA-I and II haplotypes correlate with higher levels of nAbs and a potent IFN-γ production, pointing to these alleles playing a major role in dendrimer immunity (manuscript in preparation). This approach is similar to that followed in cattle vaccinated with linear peptides, that revealed the correlation of certain MHC molecules for this species (BoLa) with a lack of protection against FMDV challenge (Garcia-Briones et al., 2000).

In previous experiments, pigs had been immunized with a 2 mg/dose of dendrimer peptide. Considering that an important feature for vaccine manufacturing is reducing the cost of large-scale production, lowering the amount of the dendrimers still capable to induce optimal anti-FMDV immune responses was explored during this Thesis.

Memory is a hallmark for vaccines, including FMDV ones. Therefore, an important issue is not only the induction of a potent immune response, but also the duration of anti-FMDV immunity is also crucial. Conventional vaccines need re-immunization shots every 6 months to maintain protective and long-term immunity and only high potency vaccines, which incorporate around one hundred fold the protective dose included in conventional ones, can protect beyond this time (Cox et al., 2003; Salt et al., 1998). Thus, long-term protective responses induced by alternative immunogens is mandatory for next-

generation FMDV vaccines (Blanco et al., 2017; de Los Santos et al., 2018). For these reasons, another main topic of this Thesis was to analyze the duration of the immune response of dendrimer peptides in a natural FMDV host.

6.2.1 The peptide B_2T-TB_2 as a promising new dendrimer vaccine candidate

The results obtained in mice with the dendrimer B_2T-TB_2 prompted us to study the immunogenicity of this construction in pigs with emphasis in: i) exploring the effect of lowering the amount of dendrimer peptide on the immune responses, and ii) analysis of the duration of anti-FMDV immunity. For comparative responses, pigs immunized with B_2T-3A were included in the experiment. Immunization with two doses of peptides B_2T-3A (2 mg) and B_2T-TB_2 (0.5 or 2mg) induced long term antibodies (up to 5 months pi) detected by ELISA as well as high levels of nAbs. Notably, both constructions elicited detectable levels of nAbs as soon as day 14 pi. The nAbs titers before boost (day 28 pi) were higher in the two groups vaccinated with B_2T-TB_2 than in those of the B_2T-3A group, being this difference also noticed after boost at day 154 pi (5 months pi), indicating that the antibodies induced by B_2T-TB_2 were lasted longer than those of the B_2T-3A group (**Figure 10**). These data highlight the importance of multiepitope vaccine platform, in which an elevated density of B-cell epitopes in an antigen correlates with the magnitude of the immune response (Bachmann et al., 1993; Zinkernagel, 2018). Previous results from our group showed that dendrimer B_4T, encompassing four copies of the B-cell epitope (tetravalent) did not improve the immunogenicity of the bivalent B_2T-3A for type O FMDV (Blanco et al., 2016). However, as commented in previous section, although the amount of B-cell epitopes in each molecule is four in both constructions, the way they are display in the vaccine platform, tightly grouped at one side of the molecule (B_2T) versus splitted at opposite sites (B_2T-TB_2), can influence their immunogenicity (Blanco et al., 2013; Monso et al., 2013) (Forner, Thesis 2020) (see section 6.1).

The induction of antibodies capable of efficiently opsonize FMDV for subsequent phagocytosis and viral clearance, has been described as another effective antibody-mediated mechanism relevant in protection (McCullough et al., 1988). For this reason, the determination of isotype-specific antibodies is relevant for FMDV vaccine research. In this line, results presented in this Thesis show high IgG1 and IgG2 titers in the pigs immunized with each of the two dendrimer peptides. Only two animals (pigs 89 and 90) immunized with 0.5 mg of B_2T-TB_2 elicited lower titers (**Figure 11**), although the differences were non-statistically significant. These results suggest that the dimeric

molecule B_2T-TB_2 does not influence/modify the class-switching induced by B_2T-3A. It is known that IgG2 isotype efficiently opsonizes FMDV and activates the complement cascade, playing a major role in FMDV protective immunity. The strong IgG2 induction observed is in line with those previously reported for other dendrimer peptides by our group (Blanco et al., 2016; Cubillos et al., 2008) and with human adenovirus expressing FMDV capsid proteins (Mayr et al., 1999). On the other hand, IgG1 induction is as well important, since a bias towards this isotype has been related to protection in cattle (Capozzo et al., 2011; Grant et al., 2016; Sitt et al., 2019; Soria et al., 2018). Moreover, class switching towards IgG2 has been correlated with an increment of IFN-γ production in other viral infections, which associates with effective antiviral immunity, an important consideration for vaccine development (Hangartner et al., 2006).

The induction of IgG2 biased response is also supported by the strong IFN-γ production observed upon dendrimer-immunization (**Figure 12**). A consistent primary response of IFN-γ expressing T-cells was observed on pigs after the first dose of peptides B_2T-3A and B_2T-TB_2, as reported previously for B_2T-3A and other related dendrimers (Blanco et al., 2016; Cubillos et al., 2008). Upon the peptide boost, and in contrast to what observed with antibodies, no significant increase in IFN-γ expressing T-cells was noticed. This lack of re-stimulation, also observed in other experiments of this Thesis (see sections 5.4.2 and 5.5.4), could be due to the temporal gap between the second immunization and sample collection (caused by logistic constrains) that impaired detection of an earlier T-cell boosting. In any case, while antibody response is sustained, a limited expansion of IFN-γ producing cells is observed upon vaccine boost. A possible explanation comes from studies with prime-boost vaccination regimes, in which early re-immunization can lead to suboptimal T-cell responses and diminished proliferation potential of T-cells, since sufficient time (around 2-3 months) after a priming is recommended to induce optimal memory T-cell responses (Sallusto et al., 2010). However, because of logistic issues, we were not able to perform this type of priming and long-term boosting experiments at INIA facilities. Likewise, it has been documented that a DNA-based FMDV vaccine did not increment IFN-γ production upon re-immunization (Kotla et al., 2016). A lack of memory T-cell activation has also been documented by Talker and co-workers (Talker et al., 2015) showing that after a secondary swine influenza virus antigen recall, a deficient $CD4^+$ T-cell expansion, reflected in low frequencies of IFN-γ producing PBMCs, was observed.

The authors suggested that this phenotype could be due to a masking effect exerted by T-cells to other antigens (Talker et al., 2015).

Assuming that additional studies with a higher number of animals will be necessary to further confirm their statistical significance, our results support that a long-lasting specific immune response elicited by B_2T-3A associated with reduced susceptibility to FMDV infection (not included in this memory) was observed at least 4.5 months after the last immunization (Cañas-Arranz et al., accepted for publication). Although challenge experiments need to be performed, the promising long-term immune response elicited by B_2T-TB_2 resembling those of B_2T-3A would presage similar results. This duration of the protective immunity (about 4.5 months) is close to those elicited by conventional vaccines (Robinson et al., 2016) and to that recently reported for cattle immunized with a deficient human adenovirus vector expressing FMDV empty capsids (Sitt et al., 2019). Indeed, in some FMD-endemic regions, *e.g.*, South America, manufacturers are required to show that oil-adjuvated conventional vaccines are able to induce protective antibody levels for 180 days after one dose and for 1 year after revaccination, performed 6 months after the first dose (Smitsaart and Bergmann, 2017).

Despite the promising results with B_2T-TB_2, the synthesis of this construction remains less efficient than that of the other B_2T- derivatives, including B_2T-3A. Thus, further work on the optimization of the strategy for B_2T-TB_2 synthesis is required before this dendrimer can be considered as a feasible candidate, in terms of costs, for commercial vaccines formulation. Moreover, this B_2T-TB_2 platform would allow the incorporation of B-cell epitopes from two different serotypes, rendering this peptide an attractive vaccine candidate. However, these current synthesis limitations focused us on the characterization of the protective immune response elicited in swine by B_2T-3A and new related dendrimeric peptides.

6.2.2 A single dose of dendrimer B_2T-3A can protect pigs from FMDV challenge

Protection after a single inoculation is a must for effective FMD vaccines, as it reduces both the cost of the vaccine as well as the logistics and labor expenses associated with double immunization schedules. The application of a single dose vaccination program to the pig population can favor disease eradication, considering that pigs serve as amplifiers of FMDV. Therefore, a rapid control of the infection in swine is essential for disease control (Perez and Willeberg, 2017). This is particularly relevant in settings such as

diseases outbreaks occurring in areas where FMD is not enzootic and livestock remains unvaccinated (Smitsaart and Bergmann, 2017). To our knowledge, with the exception of adenovirus-vectored vaccines expressing FMDV empty capsids (Pacheco et al., 2005), few studies exist on the capability of FMD subunit vaccines alternative to classical ones to confer protection in relevant hosts after a single administration (Lyons et al., 2019). In this context, our exploration of the minimal amount of B_2T-3A dendrimer still conferring protection is pertinent, both for understanding the response mechanisms to B_2T-3A, as well as for scaling down production costs, a relevant issue for FMD vaccine development. Our group had previously shown that a two-dose immunization with dendrimer B_2T-3A afforded solid and long lasting protection against FMDV (Cañas-Arranz et al., submitted and (Blanco et al., 2016)).

To explore the feasibility of B_2T-3A as a protective, single dose subunit vaccine alternative to conventional FMD formulations, the original 2 mg dose and a four-fold lower one (0.5 mg) have been compared. The challenge protocol followed in our experiment was as recommended by the OIE manual, except that virus was inoculated 3 days earlier than indicated (OIE, 2018). Under such conditions, full protection, considered as the absence of lesions at points other than the inoculation site, was observed in 80% of pigs vaccinated with B_2T-3A; 3 out of 5 in the 2 mg dose group, and 4 out of 5 in the 0.5 mg group. This level of protection correlated with an average increase in nAb that was not observed for B-specific IgG antibodies detected by ELISA (**Figure 14**), suggesting the recall upon viral infection of a subset of FMDV-specific memory B-cells, as well as of memory T-cells capable to promote B-cell maturation. This increment of nAb in protected animals, matching that of a non-immunized pig, might reflect virus multiplication to a limited extent not resulting in detectable viremia (**Table 6**). Similar levels of nAb boost upon virus challenge have been reported in pigs and cattle immunized with live-vectored vaccines or FMDV-like particles (Guo et al., 2013; Sitt et al., 2019).

Non-protected (pigs 3 and 10) and the partially protected (pig 5) animal showed lower levels of nAb at the day of challenge, albeit animals with lower VNT yet protected were also observed (**Figure 14B**); the lack of correlation in a fraction of protected animals immunized with different FMDV vaccines has been described (McCullough et al., 1992a; Taboga et al., 1997).

In addition, and supporting T-3A recognition as a T-cell epitope, specific IFN-γ releasing activated T-cells were detected rather early, at day 15 pi, in 7 of the 10 immunized pigs.

The average frequencies of IFN-γ releasing cells in PBMCs from pigs in the 0.5 mg dose group were higher than those in the 2 mg group (**Figure 15**). These surprising results have been reported as well for conventional vaccines by Oh and co-workers, who described that ¼ dose of an inactivated vaccine induced more IFN-γ than a full dose in cattle (Oh et al., 2012). This would suggest a dose-independent IFN-γ expression upon FMDV antigen recall. Moreover, differences in the *in vitro* dose-effect of peptide stimulation and/or on the MHC allele composition of the animals analyzed could also explain these results. One animal that was low responder at day 15 pi (pig 7), turned out to be protected upon challenge and pig 5, also low responder, was partially protected. Among responder animals, no clear correlation between IFN-γ releasing cell frequency at day 15 pi and protection to FMDV challenge was observed, *i.e.* non-protected pigs 3 and 10 showed high responses. Thus, as previously reported VNT and T-cell stimulation do not fully correlate with the protection conferred by FMD peptide vaccines (Taboga et al., 1997).

Our evidence of the consistent protection with a single B_2T-3A dose upon challenge at day 25 pi is particularly remarkable with regard to the 0.5 mg dose group, as it opens the way to significant savings in manufacturing costs. Indeed, as commented in section 6.1, immunization of a limited number of pigs with two doses of B_2T-3A conferred a long-lasting reduced susceptibility to FMDV infection, up to 136 days (19/20 weeks) post-boost. Remarkably, a similar duration of the protective response was achieved by a single dose of this peptide (data not shown in this Thesis) (Cañas-Arranz et al., submitted).

In summary, our findings portray the B_2T-3A peptide as a safe, potentially cost-effective candidate to be included in FMD vaccine formulations conferring single-shot protection.

6.2.3 Immunogenicity of dendrimers harboring the epitope T-3D: B_2T-3D, B_2T-3A3D and B_2T-3D3A

The protective responses elicited by B_2T-3A and other related dendrimeric constructs, associate with the induction of high titers of nAb, and activation of lymphocytes capable of providing T-cell help for effective production of nAbs (Blanco et al., 2016; Cubillos et al., 2008) (and this Thesis). Besides, such T helper epitopes can also stimulate T-cell subsets leading to the expression of IFN-γ, a cytokine with a relevant role in the antiviral response (McCullough and Sobrino, 2004). Thus, a further characterization of the functional role of the T-cell epitope(s) recognized by swine lymphocytes in the B_2T dendrimers is relevant to understand how they work and to design vaccine improvements. Most of the animals immunized with B_2T-3A elicited, in previous experiments and in

those perform during this Thesis, high levels of nAbs and activated T-cells, supporting the criteria of being widely recognized as T helper epitopes by pigs belonging to Spanish breedings. Nevertheless, testing of FMDV-specific epitopes functionally analogous to T-3A is relevant to extend the repertoire of T-cell epitopes to be included in dendrimeric vaccines to cope with SLA polymorphisms of different pig breedings, as well as to explore potential differences in the quality of the responses they elicit.

Replacement of T-3A or its combination with other T-cell peptides are possibilities to explore the effect of altering the recognition B_2T constructions by T- cells. As commented above, different T-cell epitopes previously identified in swine were not efficiently recognized by murine lymphocytes. Thus, despite the limited amount of nAbs elicited by B_2T-3D in mice, we selected T-3D to study the effect of its inclusion on the immunogenicity of B_2T dendrimer in swine. In addition, a control not yet studied in previous experiments, B_2 −a version of B_2T lacking the T-cell peptide−was included to allow dissection of the effector components of the B_2T-dendrimeric peptides in eliciting B- and T-cell responses. In these experiments, B_2T-3D and B_2T-3A induced similar nAbs titers in the pigs, and when combined in the same molecule, the orientation used−B_2T-3A3D or B_2T-3D3A, echoed on the magnitude of the response, resulting B_2T-3A3D the one that elicited optimal nAb titers, especially after peptide boost, contrary to B_2T-3D3A. These results evidence differences in the immunomodulation of the antibody response to the B-cell epitope. A similar influence of the relative orientation between the B and the T-cell epitope on nAb production was described in pig for linear peptides spanning the VP1 (140-160) B-cell epitope and T-3A (Blanco et al., 2013).

Interestingly, the lack of induction of total antibodies (determined by ELISA) and nAbs in pigs immunized with B_2 is consistent with the need of specific T-cell activation for induction of antibodies against peptide B (*i.e* GH-loop). Nevertheless, the influence of other factors such as the acquisition in the B_2 molecule of non-functional conformations of the B-cell epitope resulting in impaired presentation to B- and/or T-cells cannot be excluded. Interestingly, B_2T-3A also induced IFN-γ expressing T-cells that were *in vitro* recalled by both T-3A peptide and to a lesser extent by B-peptide with similar time courses, supporting that both sequences were recognized as T-cell epitopes. Conversely, the IFN-γ expressing cells elicited by B_2T-3D preferentially recognized the T-3D peptide, supporting its dominance.

When the two epitopes were combined in a single molecule, B_2T-3A3D and B_2T-3D3A induced a clear activation of specific T-cells. In fact, when peptides with each of the T-cell epitopes (T-3A and T-3D) were used as stimulators, a marked hierarchy of T-cell responses were observed, being peptide T-3D the major inducer of IFN-γ. The mechanisms underlying this observation or, in other words, the reasons why peptide T-3D is immunodominant over the other epitopes (T-3A and B) remains to be clarified.

The determinants rendering MHC-peptide complex engagement and their ability to activate T-cells are varied and complex (Marrack et al., 2008). It is well known that the requirements for MHC-I anchoring is more restrictive than for MHC-II. MHC-I molecules are able to display peptides from 8 to 10 amino acid, whereas MHC-II are able to present peptides from 12 to 16 amino acid in length (Bjorkman et al., 1987; Scott et al., 1998). Moreover, the correct intracellular processing and trafficking of the peptide, the subsequent MHC/peptide complex formation and exposure on the cell surface and engagement with the T-cell receptor (TCR) are also critical in eliciting the response (Blum et al., 2013).

Regarding antigen processing, B_2-T dendrimers incorporates a putative cathepsin D cleavage site (Lys-Lys) adjacent to the N-terminus of the T-cell peptide (**Table 3**). Cathepsin D is a protease known to operate in the late endosome, participating in protein cleavage for later MHC-II-peptide complex formation (Blum et al., 2013). We have so far no direct experimental evidences that the dendrimers are being processed, although *in vitro* experiments to address this point are scheduled. Other factors such as the specificity of the proteasome, ER aminopeptidases1 (ERAP1), and the transporter associated with antigen processing (TAP) for MHC-I or antigen unfolding and proteolysis for MHC-II, influence processing and antigen presentation (Blum et al., 2013). Once on the cell surface, the TCR and MHC/peptide complex engagement is more critical than the anchoring peptide to MHC molecules, since a single TCR must react with a MHC-peptide-bound complex to activate the T-cell. Unfortunately, any of the inbred mice with MHC-defined antigens tested so far in our lab were responders to the dendrimers (data not shown), impairing, for the moment, the use of a murine model to study the cellular and molecular immunology of the response they elicit. As an alternative, we are exploring the possibility of performing SLA/peptide binding assays in cell lines expressing tetramers of SLA molecules derived from inbred pig strains that are responders to our dendrimeric peptides.

In general, the inclusion of different T-cell epitopes in subunit vaccines, such as the dendrimeric peptides analyzed here, increases the chances of T-cell epitope-MHC interaction and can expand the subsequent response to these vaccines in different individuals or species. Indeed, inclusion of a non-redundant variety of T-cell epitopes, a strategy followed in diverse multi-epitope vaccine approaches, can also allow targeting to different T-cells subsets that contribute to immunity (Lennerz et al., 2014; Zhang, 2018; Zhang et al., 2015).

Regarding the isotype profile of the antibody response, B_2T-3A3D induced high IgG1 and IgG2 titers similar than those of B_2T-3D, suggesting that peptide T-3D was dominating the IgG class-switching as observed in the induction of IFN-γ expressing T-cells. On the other hand, B_2T-3D3A induced lower titers of both IgG isotypes, suggesting that the dominant effect of T-3D is dependent on the orientation of the epitopes in the dendrimer.

Overall, addressing the protection afforded by these dendrimer peptides emerges as an interesting option that will be eventually assessed in the near future.

6.2.4 Characterization of T-cell population activated in dendrimer-immunized pigs

In order to further characterize the T-cell populations involved in the response to the dendrimeric peptides harboring different T- cell peptide combinations, flow cytometry analyses of PBMCs from immunized animals were performed. In humans and mice, it is well established that IFN-γ is produced by T lymphocytes and NK cells. The situation in swine is less clear, reflecting the complexity of the cell subsets involved in this process and the limited availability of specific reagents for the characterization of swine antigens (Gerner et al., 2015; Talker et al., 2013). Thus, a wide variety of IFN-γ producing cells are known to be induced by specific stimuli or cytokines, including Th-cells, Tc-cells, NK cells and γδ T-cells, being the latter highly abundant in swine (Gerner et al., 2009; Pintaric et al., 2008). The percentage of single positive populations $CD4^+$ and $CD8\text{-}\beta^+$ derived from PBMCs found in the pigs analyzed, agreed with those described for outbred 3-5 months-old pig populations (Zuckermann and Husmann, 1996). In our experiments, a strong IFN-γ production was noticed in $CD4^+$ subset derived from vaccinated animals upon homologous stimulation, ranging from 3-5 %, depending on the peptide. Although a limited number of animals were used for the analysis (n = 2), B_2T-3D induced the highest IFN-γ secretion (**Figure 24**). These results correlated with those of the induction

of IFN-γ secreting cells and point to the epitope T-3D as a potent IFN-γ inductor, as well as mainly recognized by $CD4^+$ cells.

In contrast, a limited activation of the cytolytic CD8-β^+ subset was noticed in these experiments (ranging from < 1- 1.5 % depending on the peptide), suggesting a minor contribution of CD8-β^+ cells in the response to the dendrimers. These results are consistent with the kind of T-cell epitopes included in the dendrimers, as they were previously identified as swine T helper epitopes (Blanco et al., 2001; Garcia-Briones et al., 2004). The possibility of eliciting heterotypic CD8 responses against highly conserved FMDV epitopes can interestingly contribute to potentiate the protective responses against this highly antigenic variable virus. Induction of CD8 responses has been described upon vaccination of pigs (Fernandez-Sainz et al., 2019; Patch et al., 2011) and cattle (Guzman et al., 2010; Guzman et al., 2008). Therefore, further understanding of the role that CD8 responses can play in the protection conferred by the B_2T peptides, as well as the development of strategies aimed at increasing activation of CD8 T-cells, are among the tools for improving dendrimeric peptide vaccines.

The major role of the $CD4^+$ cell subset on the response to dendrimer B_2T-3D was confirmed when this population was magnetically depleted from PBMCs. Eluted $CD4^-$ fraction subset failed to produce IFN-γ whereas enriched $CD4^+$ fraction substantially produced IFN-γ upon dendrimer and T-peptide stimulation (**Figure 25**). Although the fractioning efficiency of PBMCs was very high, it cannot be ruled out that very few APCs present in $CD4^+$ fraction could initiate $CD4^+$ T-cell activation. In fact, cell staining revealed around 4 % of $CD4^+$ T-cell fraction expressing CD172a, a marker for APCs (see Supplemental Information 8.2).

Two major conclusions can be drawn from this experiment: i) $CD4^+$ T-cells are the major subset involved in T-cell immune responses upon dendrimer vaccination, thereby a strong $CD4^+$-dependent response is induced, and ii) T-3D is a promiscuous porcine T helper epitope since ablation of cytokine release was observed in $CD4^-$ subset upon stimulation with the T -peptide.

The importance of $CD4^+$ T-cells upon FMDV vaccination has been address by Naesssens and co-workers, who demonstrated that depletion of this subset *in vivo* ablated antigen-dependent lymphoproliferative responses (Naessens et al., 1998). On the other hand, a $CD4^+$ T-cell-independent nAb response has been reported using in vivo depletion of this

cell subset in a different species (Juleff et al., 2009). In any case, our results point that porcine $CD4^+$ T-cells are critical for effective T-cell anti-FMDV immunity.

A fraction of primed T-cells can persist in the absence of specific antigen (memory T-cells) being necessary for a rapid response upon antigen recall (Gray, 2002; Sallusto et al., 2010; Zinkernagel, 2003). The porcine 2E3 is a marker that discriminates two functionally different subsets of $CD4^+$ T-cells: naive $CD4^+2E3^+$ and effector/memory $CD4^+2E3^-$ cells (Revilla et al., 2004). Considering that the PBMCs used for the enrichment studies were collected at 3.5 months pi, the presence in them of a substantial proportion of memory T-cells was expected. The relative percentage of $CD4^+2E3^+$ and $CD4^+2E3^-$ in pigs immunized with each of the four dendrimer peptides (**Figure 26**) was consistent with those reported for 14-20 months old Large-White outbred pigs (Revilla et al., 2005). In order to discern the phenotype of activated T-cells upon homologous dendrimer stimulation, the expression of IFN-γ was analyzed in both cell subsets. The results showed a mild expression of IFN-γ in the $CD4^+2E3^+$ subset whereas a strong cytokine detection was observed in the $CD4^+2E3^-$ one, suggesting that effector/memory T-cells were responsible of responses in the two animals analyzed from each peptide group. This effect was more prominent for B_2T-3D immunized animals and correlated with previous results in the ELISPOTs assays (**Figure 18**), further supporting the capacity of T-3D in eliciting potent Th1 and IFN-γ responses. Moreover, IFN-γ detection in both subsets in B_2T-3D3A immunized pigs, was barley observed with levels similar to that of the B_2-immunized control animal, suggesting, again, that T-3D3A orientation is less efficient in T-cell activation. Overall, our results provided evidences of the activation of effector/memory T-cells upon immunization with the B_2T dendrimers.

Different subsets of memory T-cells can be functionally distinguished and, studies in mice and humans have revealed that these cells survive in different stages of differentiation, such us central memory T-cells (T_{CM}) or effector memory T-cells (T_{EM}) (Reinhardt et al., 2001; Sallusto et al., 1999). Unfortunately, in the pig, some of the analogous subsets remain poorly characterized (Gerner et al., 2009; Reutner et al., 2012). Therefore, further studies are required to identify specific T-cell subset(s) being activated upon peptide immunization.

As commented above, not only IFN-γ, but other pro-inflammatory cytokines such us TNF-α or IL-2 can drive T-cells towards a Th1 pathway, the main effector cells leading for viral clearance. Therefore, the expression of double positive cytokine (IFN-γ and

TNF-α) in the $CD4^+$ subset of pigs immunized with each of the four dendrimer peptides was analyzed. The results obtained showed that the majority of $CD4^+$ cells produced both cytokines and that a minor subset of $CD4^+$ were single producers (**Figure 27**). This is in agreement with previous results by Revilla and coworkers, who proved that most of the memory T-cells secrete both cytokines (Revilla et al., 2005). Although the analyses of the expression of IL-2 and the 2E3 marker would complement our conclusions, these results point to the induction by the dendrimeric peptides of Th1 effector responses. Interestingly, low levels of $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T-cells were observed in B_2T-3D3A group that were similar to that in the B_2-immunized pig, indicating again, that the T-cell orientation T-3D3A elicited lower T-cell responses (**Figure 27**). To our knowledge, this is the first study that addresses in detail the T-cell subsets stimulated upon FMDV immunization with either the whole virus or its subunits.

Furthermore, as shown in **Figure 24**, B_2 peptide was not able to induce IFN-γ in $CD4^+$ subsets, strengthening the importance of the incorporation of a specific T-cell epitope for the capacity of the dendrimers to elicit IFN-γ secreting T cells. It cannot be excluded that B_2 peptide could elicit non-Th1 cytokines, not analyzed here. In any case, the role of non-Th1 cytokines in FMDV protective immune responses is likely to be limited.

Altogether, our results point to Th1 responses as the main effector mechanism of the T-cell activation elicited by the dendrimeric constructs. Detection of other cytokines involved in Th1 responses such as IL-2 or IL-12 or Th1-restricted transcription factors such as T-bet, will contribute to confirm this observation.

6.3 Dendrimer peptides as a valuable FMDV vaccine: crossneutralization of sera from peptide-immunized pigs and immunogenicity of mixtures of serotype-specific B_2T-3A versions

Implementation of efficient vaccination campaigns against FMD, requires the use of inactivated viruses capable of eliciting protective responses against circulating and emerging FMDVs, including serotype specific vaccine isolates into vaccine formulations (Sobrino and Domingo, 2001). Thus, because of the wide antigenic range presented by FMDV, an optimal vaccine needs to protect against a wide FMDV spectrum. This is particularly the case for vaccines against type O viruses, which are responsible of major outbreaks in epidemic countries (Knowles and Samuel, 2003; Knowles et al., 2005).

Initial experiments with lineal peptides indicated that the nAbs elicited were able to neutralize not only the homologous virus, whose sequence was that of the VP1 GH-loop they contained, but also heterologous FMDV isolates (Parry et al., 1989). To test this possibility, the neutralization capacity of sera from dendrimer peptide-immunized pigs from the different experiments performed in this Thesis, was determined using different type O FMDV isolates. Interestingly, dendrimer peptides elicited, in most cases, high titers of cross-neutralizing antibodies, which, for some isolates, were higher than those against the homologous virus (**Figure 28**). Factors inherent to the assay used, such as differences in thermal stability among the viral isolates analyzed, can contribute to explain these observations, which have also been reported for sera from type A FMDV conventionally vaccinated animals (Bari et al., 2014) and an adenovirus-vectored type O FMDV vaccine (Fernandez-Sainz et al., 2017).

B_2T-like platforms, allow incorporation of B-cell epitopes from different FMDV isolates (including those of different serotypes), potentially expanding the spectrum of the anti-FMDV immunity they produced. Accordingly, we also investigated the potential of B_2T dendrimers to elicit heterotypic responses, by assessing the immunogenicity in mice of non-covalent mixtures of two B_2T-3A variants in which the B-cell epitope belonged to either type O or type C FMDV. High titers of nAbs were detected after boosting with the peptide mixture and a strong correlation was observed between both titers (**Figure 30C**). Moreover, these nAb titers were similar to those elicited by each of the single B_2T-3A peptide, highlighting that B_2T is a valuable platform to elicit heterotypic FMDV nAbs. Experiments in pigs including viral challenge with both serotypes are required to confirm the potential of non-covalent mixtures of B_2T-3A as heterotypic vaccines.

6.4 Effect of preventing the attachment of B_2T-3A construction to integrins

Integrins are the main FMDV receptors an are widely expressed in many mammalian cell types (Jackson et al., 2003; Jackson et al., 2000). The B-cell epitope encompassed in the dendrimers studied in this Thesis, contains the RGD motif that is the integrin-binding site of FMDV (see Introduction 2.2). We speculated that the attachment of our peptides to non-professional immune cells could be detrimental in eliciting anti-FMDV responses, as it could decrease the amount of dendrimer available to interact with APCs to initiate its processing and presentation to T-cells. If so, avoiding interaction of peptides with integrins, would target them more rapidly and/or efficiently to APCs.

To attempt confirming this hypothesis, serotype C versions of B_2T-3A peptide were synthesized including replacements at the RGD motif (RGD →HGD or RGD→KGD) previously reported to impair integrin binding but not the virus capability to be neutralized by mAb (Verdaguer et al., 1998). Each of these B_2T-3A versions showed a reduced productive binding to susceptible cells and were able to elicit nAbs (**Figure 32**). The irrelevant peptide T-3A showed mild inhibition of infection at 25 µM concentration. As the FMDV NSP 3A associates with cellular membranes *in vitro* (Gonzalez-Magaldi Thesis, 2012), it cannot be excluded that T-3A peptide could be interfering in virus-cell attachment, and subsequent infection. Contrary to what initially expected, when compared with B_2T-3A peptide a trend towards a lower induction of VNT was observed in animals immunized with the variant peptide versions, which was statistically significant for B_2T (R141H) upon peptide boost. Although alterations in the immunogenicity of the B-cell epitope in the variant dendrimers, due to the replacements introduced, cannot be ruled out, one possible explanation for this result is that the impairment of the binding capacity of B_2T-3A to integrins could downregulate its presentation by DCs. Indeed, integrins have been shown to be relevant for FMDV uptake by DCs (McCullough et al., 2017).

6.5 The future of FMD vaccines: peptides in the spotlight

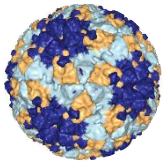
Despite the advances in understanding its biology and the multiple approaches explored for its control, FMDV still remains as a global threat and seeking for an affordable and disadvantage-free protective FMDV vaccine remains a challenging issue. Among the immunization strategies pursued, peptide vaccines fulfill the requirements for FMD vaccine approval, and as addressed in the Introduction (2.5.5) and shown in this Thesis, they can confer protection to natural hosts.

Nevertheless, FMDV infects a wide range of species, and their populations are highly variable endowing the virus with a high adaptability, *i.e.* the easy selection of antibody escape mutants. These characteristics, as occur with other RNA viruses, hamper the development of effective subunit vaccines, such as peptide vaccines, in which a limited number of epitopes is included.

Despite similar challenges, peptide vaccines have already been shown to be effective against different cancer diseases and other infectious diseases (Combadiere et al., 2019; Lamonaca et al., 1999; Lennerz et al., 2014; Li et al., 2014) and their value should not be underestimated. Among the advantages of peptides, are: i) the multimerization

approaches, which allow the incorporation of multiple epitopes to afford immunity, ii) mimicking highly density antigens in a single molecule and iii) avoiding antigens that could be detrimental in protective responses.

The data presented in this Thesis on the effectiveness, the mechanisms behind and the duration of the protective response elicited by FMDV dendrimeric peptides can be considered as a proof of concept for the extension of analogous strategies to different FMDVs and host species.



CONCLUSIONS / CONCLUSIONES

7. Conclusions

1. The analysis of the immunogenicity in mice of derivatives of the dendrimeric prototype B_2T-3A have revealed that its dimeric version B_2T-TB_2, is the only construction that elicit IgG and neutralizing antibody levels higher than B_2T-3A.

2. The constructions in which the T-cell epitope T-3A is replaced by other porcine T cell epitopes induce lower antibody responses, highlighting the limitations of the mouse as a screening model for B_2T FMDV dendrimers.

3. The long-lasting immune response, reflected in both neutralizing antibodies and IFN-γ expressing cells, induced by two doses of peptide B_2T-3A (2 mg each) or B_2T-TB_2 (2 mg or 0.5 mg) in an important FMDV host (the pig), strengthens the value of these peptides as feasible FMD vaccine candidates.

4. A single immunization with B_2T-3A with a dose of 2 mg or 0.5 mg can afford solid protection upon homologous FMDV challenge, supporting the use of this peptide as a possible cost-effective alternative FMD vaccine.

5. The dendrimer peptide B_2T-3D, encompassing a T-cell epitope different from that in B_2T-3A, and versions harboring both, T-3A and T-3D, in the two possible orientations (B_2T-3A3D and B_2T-3D3A), elicit high neutralizing antibody titers in pigs. T-3D was shown to be immunodominant T-cell epitope since it evoked levels of IFN-γ expressing cells comparing T-3A in the *in vitro* recall assay.

6. In pigs immunized with dendrimeric peptides, $CD4^+$ T-cells are the major subset involved in IFN-γ expression upon *in vitro* recall. Depletion of $CD4^+$ cells from PBMCs abolished the production of this cytokine. The majority of $CD4^+$IFN-γ^+ cells showed a memory ($CD4^+2E3^-$) and a multifunctional phenotype, as they expressed IFN-γ and TNF-α, suggesting that the peptides induced a potent Th1 pro-inflammatory response.

7. Non-covalent mixtures of B_2T-3A in which the sequence of the B-cell peptide belonged to viruses of two different FMDV serotypes elicit high levels of cross-reactive neutralizing antibodies against, supporting the potential of this approach to extend the protective spectrum conferred by dendrimer peptides

8. The overall results presented support the use of the dendrimer platforms, such as those studied here, as potential FMDV heterotypic next-generation vaccines.

7. Conclusiones

1. El análisis en ratón de la inmunogenicidad de derivados del dendrímero prototipo B_2T-3A ha revelado que su versión dimérica, B_2T-TB_2, es la única que induce títulos de IgG específica y de anticuerpos neutralizantes mayores que los observados para B_2T-3A.

2. Las construcciones en las que T-3A es reemplazado por otros epítopos T porcinos, inducen menores respuestas de anticuerpos, lo que subraya las limitaciones del modelo de ratón para el *screening* de dendrímeros B_2T de VFA.

3.La respuesta de anticuerpos neutralizantes y células productoras de IFN-γ inducida por dos dosis del dendrímero B_2T-3A (2 mg cada una) o B_2T-TB_2 (2 mg ó 0.5 mg) en un importante hospedador natural de VFA (el cerdo), es duradera. Esto apoya el interés de estos péptidos como candidatos vacunales viables frente a VFA en una importante especie ganadera.

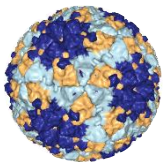
4. Una única inmunización con B_2T-3A con una dosis de 2 mg ó 0.5 mg es capaz de proteger frente al desafío experimental con VFA homólogo, apoyando el uso de este péptido como posible alternativa vacunal rentable.

5. El dendrímero B_2T-3D, que contiene un epítopo T diferente al de B_2T-3A, y verisones con ambos, T-3A y T-3D, en las dos posibles orientaciones (B_2T-3A3D o B_2T-3D3A), indujeron altos títulos de anticuerpos neutralizantes. Las respuestas T detectadas, mostraron a T-3D como un epítopo potente e inmunodominante, ya que indujo una fuerte expresión de IFN-γ en comparación a T-3A.

6. En los cerdos inmunizados con los péptidos dendriméricos, las células T-$CD4^+$ son la principal población implicada en la expresión de IFN-γ. La depleción de células $CD4^+$ de células mononucleares de sangre periférica, anuló la producción de esta citoquina. La mayoría de células $CD4^+$IFN-γ^+, mostraron un fenotipo de memoria ($CD4^+2E3^-$) y multifuncional, ya que expresaban conjuntamente IFN-γ y TNF-α, sugiriendo que los péptidos inducían una potente respuesta pro-inflamatoria Th1.

7. Mezclas no covalentes de dos péptidos B_2T-3A en los que la secuencia del epítopo B pertenecía a virus de distinto serotipo, indujeron, en ratón, altos niveles de anticuerpos neutralizantes crosreactivos, lo que abre la posibilidad de su empleo de mezclas para ampliar el espectro de protección conferida por este tipo de vacunas dendriméricas.

8. Los resultados presentados, apoyan la viabilidad de formulaciones basadas en plataformas dendriméricas, coomo las aquí estudiadas, como candidatos para vacuna de nueva generación frente a la FA.



SUPPLEMENTAL INFORMATION

8. Supplemental Information

8.1 Peptides synthesis

Linear peptides were synthesized on a Prelude instrument (ProteinTechnologies, Tucson, AZ) using Fmoc SPPS protocols by Prof. D. Andreu's group (Universitat Pompeu Fabra). Peptides recognized as B- and T-cell FMDV epitopes (**Table 2**) were assembled at 0.1-mmol scale on H-Rink amide ChemMatrix® Resin of 0.5 mmol/g substitution (PCAS BioMatrix, Quebec, Canada). The peptide chain was elongated from C-terminus to N-terminus using 8-fold molar excess of Fmoc-L-amino acids, *O*-benzotri-azole-*N,N,N'N'*-tetramethyluronium hexafluorophosphate (HBTU) and double molar amount of *N,N*-diisopropylethylamine (DIEA) dissolved in *N,N*-dimethylformamide (DMF). Between the coupling steps, Fmoc-group was removed using 20% (v/v) piperidine in DMF, and stirring for 20 min. DMF washes were performed after each coupling or deprotection step. Cleavage and side chain deprotection of peptide resins was done with TFA/ water/ triisopropylsilane (95:2.5:2.5%, v/v, 90 min, r.t). Peptides were isolated by precipitation with cold diethyl ether and centrifugation (1 x 2500 rpm/ 10 min, 2 x 2500 rpm/ 5 min), then solubilized in water containing 1% (v/v) acetic acid and lyophilized. The synthetic crude peptides were analyzed by analytical RP-HPLC and LC-MS and purified by preparative RP-HPLC (collected fractions above 95% of purity).

A second generation of peptides was also synthesized in a branched (dendrimeric) fashion. This approach chemically conjugates the previous immunogenic regions from FMDV in a single molecular platform, generically named B_nT, that displays n copies of B- and one of A T-cell epitope using the multiple antigen presentation (MAP) strategy. The B_nT candidates were detailed in **Table 3**.

8.2 Expression of CD172a in each magnetically fractioned sample

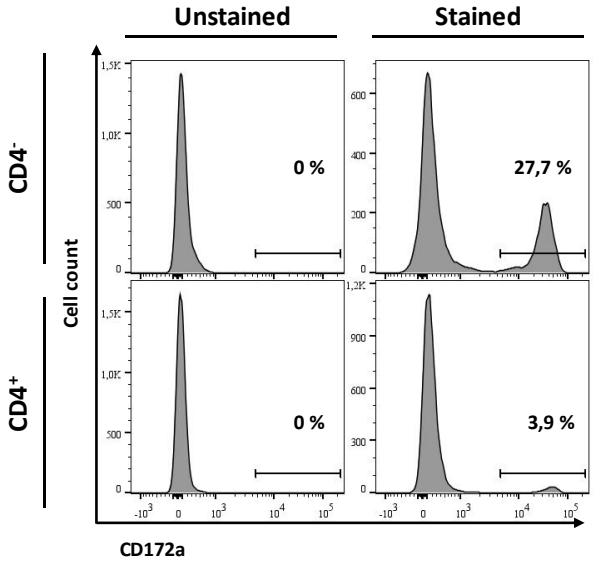
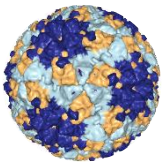


Figure S.1: The CD4$^+$ fraction contains APCs: Flow cytometry analysis was performed to determine the expression of CD172a, a marker of porcine APCs, in CD4$^+$ or CD4$^-$ fraction from fractioned PBMCs.



REFERENCES

9. References

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D., Brown, F., 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 A resolution. Nature 337, 709-716.

Alexandersen, S., Mowat, N., 2005. Foot-and-mouth disease: host range and pathogenesis. Curr Top Microbiol Immunol 288, 9-42.

Alexandersen, S., Zhang, Z., Donaldson, A.I., 2002. Aspects of the persistence of foot-and-mouth disease virus in animals--the carrier problem. Microbes Infect 4, 1099-1110.

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J Comp Pathol 129, 1-36.

Alvarez, B., Sanchez, C., Bullido, R., Marina, A., Lunney, J., Alonso, F., Ezquerra, A., Dominguez, J., 2000. A porcine cell surface receptor identified by monoclonal antibodies to SWC3 is a member of the signal regulatory protein family and associates with protein-tyrosine phosphatase SHP-1. Tissue Antigens 55, 342-351.

Andreev, D.E., Fernandez-Miragall, O., Ramajo, J., Dmitriev, S.E., Terenin, I.M., Martinez-Salas, E., Shatsky, I.N., 2007. Differential factor requirement to assemble translation initiation complexes at the alternative start codons of foot-and-mouth disease virus RNA. RNA 13, 1366-1374.

Arzt, J., Branan, M.A., Delgado, A.H., Yadav, S., Moreno-Torres, K.I., Tildesley, M.J., Stenfeldt, C., 2019. Quantitative impacts of incubation phase transmission of foot-and-mouth disease virus. Sci Rep 9, 2707.

Bachmann, M.F., Rohrer, U.H., Kundig, T.M., Burki, K., Hengartner, H., Zinkernagel, R.M., 1993. The influence of antigen organization on B cell responsiveness. Science 262, 1448-1451.

Bachrach, H.L., 1968. Foot-and-mouth disease. Annu Rev Microbiol 22, 201-244.

Bahnemann, H.G., 1972. The inactivation of foot-and-mouth disease virus by ethylenimine and propylenimine. Zentralbl Veterinarmed B 20, 356-360.

Baranowski, E., Sevilla, N., Verdaguer, N., Ruiz-Jarabo, C.M., Beck, E., Domingo, E., 1998. Multiple virulence determinants of foot-and-mouth disease virus in cell culture. J Virol 72, 6362-6372.

Bari, F.D., Parida, S., Tekleghiorghis, T., Dekker, A., Sangula, A., Reeve, R., Haydon, D.T., Paton, D.J., Mahapatra, M., 2014. Genetic and antigenic characterisation of serotype A FMD viruses from East Africa to select new vaccine strains. Vaccine 32, 5794-5800.

Barnett, P.V., Ouldridge, E.J., Rowlands, D.J., Brown, F., Parry, N.R., 1989. Neutralizing epitopes of type O foot-and-mouth disease virus. I. Identification and characterization of three functionally independent, conformational sites. J Gen Virol 70 (Pt 6), 1483-1491.

Bautista, E.M., Ferman, G.S., Golde, W.T., 2003. Induction of lymphopenia and inhibition of T cell function during acute infection of swine with foot and mouth disease virus (FMDV). Vet Immunol Immunopathol 92, 61-73.

Baxt, B., Mason, P.W., 1995. Foot-and-mouth disease virus undergoes restricted replication in macrophage cell cultures following Fc receptor-mediated adsorption. Virology 207, 503-509.

Baxt, B., Vakharia, V., Moore, D.M., Franke, A.J., Morgan, D.O., 1989. Analysis of neutralizing antigenic sites on the surface of type A12 foot-and-mouth disease virus. J Virol 63, 2143-2151.

Beard, C., Ward, G., Rieder, E., Chinsangaram, J., Grubman, M.J., Mason, P.W., 1999. Development of DNA vaccines for foot-and-mouth disease, evaluation of vaccines encoding replicating and non-replicating nucleic acids in swine. J Biotechnol 73, 243-249.

Belsham, G.J., 1992. Dual initiation sites of protein synthesis on foot-and-mouth disease virus RNA are selected following internal entry and scanning of ribosomes in vivo. EMBO J 11, 1105-1110.

Belsham, G.J., 2005. Translation and replication of FMDV RNA. Curr Top Microbiol Immunol 288, 43-70.

Berinstein, A., Tami, C., Taboga, O., Smitsaart, E., Carrillo, E., 2000. Protective immunity against foot-and-mouth disease virus induced by a recombinant vaccinia virus. Vaccine 18, 2231-2238.

Biswal, J.K., Ranjan, R., Subramaniam, S., Mohapatra, J.K., Patidar, S., Sharma, M.K., Bertram, M.R., Brito, B., Rodriguez, L.L., Pattnaik, B., Arzt, J., 2019. Genetic and antigenic variation of foot-and-mouth disease virus during persistent infection in naturally infected cattle and Asian buffalo in India. PLoS One 14, e0214832.

Bittle, J.L., Houghten, R.A., Alexander, H., Shinnick, T.M., Sutcliffe, J.G., Lerner, R.A., Rowlands, D.J., Brown, F., 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature 298, 30-33.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., Wiley, D.C., 1987. Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329, 506-512.

Blanco, E., Andreu, D., Sobrino, F. 2017. Peptide Vaccines Against Foot-and-mouth Disease, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus. Current Research and Emerging Trends. Caister Academic Press, Norfolk, UK, 317-332.

Blanco, E., Cubillos, C., Moreno, N., Barcena, J., de la Torre, B.G., Andreu, D., Sobrino, F., 2013. B epitope multiplicity and B/T epitope orientation influence immunogenicity of foot-and-mouth disease peptide vaccines. Clin Dev Immunol 2013, 475960.

Blanco, E., Garcia-Briones, M., Sanz-Parra, A., Gomes, P., De Oliveira, E., Valero, M.L., Andreu, D., Ley, V., Sobrino, F., 2001. Identification of T-cell epitopes in nonstructural proteins of foot-and-mouth disease virus. J Virol 75, 3164-3174.

Blanco, E., Guerra, B., de la Torre, B.G., Defaus, S., Dekker, A., Andreu, D., Sobrino, F., 2016. Full protection of swine against foot-and-mouth disease by a bivalent B-cell epitope dendrimer peptide. Antiviral Res 129, 74-80.

Blanco, E., McCullough, K., Summerfield, A., Fiorini, J., Andreu, D., Chiva, C., Borras, E., Barnett, P., Sobrino, F., 2000. Interspecies major histocompatibility complex-restricted Th cell epitope on foot-and-mouth disease virus capsid protein VP4. J Virol 74, 4902-4907.

Blum, J.S., Wearsch, P.A., Cresswell, P., 2013. Pathways of antigen processing. Annu Rev Immunol 31, 443-473.

Borca, M.V., Fernandez, F.M., Sadir, A.M., Braun, M., Schudel, A.A., 1986. Immune response to foot-and-mouth disease virus in a murine experimental model: effective thymus-independent primary and secondary reaction. Immunology 59, 261-267.

Borras-Cuesta, F., Fedon, Y., Petit-Camurdan, A., 1988. Enhancement of peptide immunogenicity by linear polymerization. Eur J Immunol 18, 199-202.

Borrego, B., Argilaguet, J.M., Perez-Martin, E., Dominguez, J., Perez-Filgueira, M., Escribano, J.M., Sobrino, F., Rodriguez, F., 2011. A DNA vaccine encoding foot-and-mouth disease virus B and T-cell epitopes targeted to class II swine leukocyte antigens protects pigs against viral challenge. Antiviral Res 92, 359-363.

Brito, B.P., Rodriguez, L.L., Hammond, J.M., Pinto, J., Perez, A.M., 2017. Review of the Global Distribution of Foot-and-Mouth Disease Virus from 2007 to 2014. Transbound Emerg Dis 64, 316-332.

Bronsvoort, B.M.D., Handel, I.G., Nfon, C.K., Sorensen, K.J., Malirat, V., Bergmann, I., Tanya, V.N., Morgan, K.L., 2016. Redefining the "carrier" state for foot-and-mouth disease from the dynamics of virus persistence in endemically affected cattle populations. Sci Rep 6, 29059.

Brooksby, J.B., 1982. Portraits of viruses: foot-and-mouth disease virus. Intervirology 18, 1-23.

Brown, F., Cartwright, B., 1961. Dissociation of foot-and-mouth disease virus into its nucleic acid and protein components. Nature 192, 1163-1164.

Campagnola, G., McDonald, S., Beaucourt, S., Vignuzzi, M., Peersen, O.B., 2015. Structure-function relationships underlying the replication fidelity of viral RNA-dependent RNA polymerases. J Virol 89, 275-286.

Cañas-Arranz, R., Forner, M., Defaus, S., de León, P., Bustos, M., Torres, E., Andreu, D., Sobrino, F., Blanco, E., 2020. A single Dose of Dendrimer B_2T Peptide Vaccine Partially Protects Pigs against Foot-and-Mouth Disease Virus Infection. Vaccines 8.

Capozzo, A.V., Wilda, M., Bucafusco, D., de los Angeles Lavoria, M., Franco-Mahecha, O.L., Mansilla, F.C., Perez-Filgueira, D.M., Grigera, P.R., 2011. Vesicular Stomatitis Virus glycoprotein G carrying a tandem dimer of Foot and Mouth Disease Virus antigenic site A can be used as DNA and peptide vaccine for cattle. Antiviral Res 92, 219-227.

Caron, L., Brum, M.C., Moraes, M.P., Golde, W.T., Arns, C.W., Grubman, M.J., 2005. Granulocyte-macrophage colony-stimulating factor does not increase the potency or efficacy of a foot-and-mouth disease virus subunit vaccine. Pesq. Vet. Bras. 25, 150-158.

Carrillo, C., Tulman, E.R., Delhon, G., Lu, Z., Carreno, A., Vagnozzi, A., Kutish, G.F., Rock, D.L., 2005. Comparative genomics of foot-and-mouth disease virus. J Virol 79, 6487-6504.

Cedillo-Barron, L., Foster-Cuevas, M., Belsham, G.J., Lefevre, F., Parkhouse, R.M., 2001. Induction of a protective response in swine vaccinated with DNA encoding foot-and-mouth disease virus empty capsid proteins and the 3D RNA polymerase. J Gen Virol 82, 1713-1724.

Collen, T., Dimarchi, R., Doel, T.R., 1991. A T cell epitope in VP1 of foot-and-mouth disease virus is immunodominant for vaccinated cattle. J Immunol 146, 749-755.

Combadiere, B., Beaujean, M., Chaudesaigues, C., Vieillard, V., 2019. Peptide-Based Vaccination for Antibody Responses Against HIV. Vaccines (Basel) 7.

Cortey, M., Ferretti, L., Perez-Martin, E., Zhang, F., de Klerk-Lorist, L.M., Scott, K., Freimanis, G., Seago, J., Ribeca, P., van Schalkwyk, L., Juleff, N.D., Maree, F.F., Charleston, B., 2019. Persistent Infection of African Buffalo (Syncerus caffer) with Foot-and-Mouth Disease Virus: Limited Viral Evolution and No Evidence of Antibody Neutralization Escape. J Virol 93.

Cottam, E.M., Haydon, D.T., Paton, D.J., Gloster, J., Wilesmith, J.W., Ferris, N.P., Hutchings, G.H., King, D.P., 2006. Molecular epidemiology of the foot-and-mouth disease virus outbreak in the United Kingdom in 2001. J Virol 80, 11274-11282.

Cox, S.J., Aggarwal, N., Statham, R.J., Barnett, P.V., 2003. Longevity of antibody and cytokine responses following vaccination with high potency emergency FMD vaccines. Vaccine 21, 1336-1347.

Cubillos, C., de la Torre, B.G., Barcena, J., Andreu, D., Sobrino, F., Blanco, E., 2012. Inclusion of a specific T cell epitope increases the protection conferred against foot-and-mouth disease virus in pigs by a linear peptide containing an immunodominant B cell site. Virol J 9, 66.

Cubillos, C., de la Torre, B.G., Jakab, A., Clementi, G., Borras, E., Barcena, J., Andreu, D., Sobrino, F., Blanco, E., 2008. Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. J Virol 82, 7223-7230.

Cunliffe, H.R., Blackwell, J.H., 1977. Survival of Foot-and-Mouth Disease Virus in Casein and Sodium Caseinate Produced from the Milk of Infected Cows (1). J Food Prot 40, 389-392.

Curry, S., Abrams, C.C., Fry, E., Crowther, J.C., Belsham, G.J., Stuart, D.I., King, A.M., 1995. Viral RNA modulates the acid sensitivity of foot-and-mouth disease virus capsids. J Virol 69, 430-438.

Chernyshev, A.V., Tarasov, P.A., Semianov, K.A., Nekrasov, V.M., Hoekstra, A.G., Maltsev, V.P., 2008. Erythrocyte lysis in isotonic solution of ammonium chloride: theoretical modeling and experimental verification. J Theor Biol 251, 93-107.

Childerstone, A.J., Cedillo-Baron, L., Foster-Cuevas, M., Parkhouse, R.M., 1999. Demonstration of bovine CD8+ T-cell responses to foot-and-mouth disease virus. J Gen Virol 80 (Pt 3), 663-669.

Chinsangaram, J., Mason, P.W., Grubman, M.J., 1998. Protection of swine by live and inactivated vaccines prepared from a leader proteinase-deficient serotype A12 foot-and-mouth disease virus. Vaccine 16, 1516-1522.

Chow, M., Newman, J.F., Filman, D., Hogle, J.M., Rowlands, D.J., Brown, F., 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature 327, 482-486.

De Castro, M.P., 1964. Behaviour of the foot-and-mouth disease virus in cell cultures: susceptibility of the IB-RS-2 cell line. Arq Inst Biol Sao Paulo 31, 63-78.

de Los Santos, T., Diaz-San Segundo, F., Rodriguez, L.L., 2018. The need for improved vaccines against foot-and-mouth disease. Curr Opin Virol 29, 16-25.

Diaz-San Segundo, F., Medina, G.N., Ramirez-Medina, E., Velazquez-Salinas, L., Koster, M., Grubman, M.J., de los Santos, T., 2015. Synonymous Deoptimization of Foot-and-Mouth Disease Virus Causes Attenuation In Vivo while Inducing a Strong Neutralizing Antibody Response. J Virol 90, 1298-1310.

Diaz-San Segundo, F., Salguero, F.J., de Avila, A., de Marco, M.M., Sanchez-Martin, M.A., Sevilla, N., 2006. Selective lymphocyte depletion during the early stage of the immune response to foot-and-mouth disease virus infection in swine. J Virol 80, 2369-2379.

DiMarchi, R., Brooke, G., Gale, C., Cracknell, V., Doel, T., Mowat, N., 1986. Protection of cattle against foot-and-mouth disease by a synthetic peptide. Science 232, 639-641.

Doel, T.R., Gale, C., Brooke, G., DiMarchi, R., 1988. Immunization against foot-and-mouth disease with synthetic peptides representing the C-terminal region of VP1. J Gen Virol 69 (Pt 9), 2403-2406.

Doel, T.R., Pullen, L., 1990. International bank for foot-and-mouth disease vaccine: stability studies with virus concentrates and vaccines prepared from them. Vaccine 8, 473-478.

Duque, H., Baxt, B., 2003. Foot-and-mouth disease virus receptors: comparison of bovine alpha(V) integrin utilization by type A and O viruses. J Virol 77, 2500-2511.

Eschbaumer, M., Stenfeldt, C., Smoliga, G.R., Pacheco, J.M., Rodriguez, L.L., Li, R.W., Zhu, J., Arzt, J., 2016. Transcriptomic Analysis of Persistent Infection with Foot-and-Mouth Disease Virus in Cattle Suggests Impairment of Apoptosis and Cell-Mediated Immunity in the Nasopharynx. PLoS One 11, e0162750.

Fernandez-Sainz, I., Gavitt, T.D., Koster, M., Ramirez-Medina, E., Rodriguez, Y.Y., Wu, P., Silbart, L.K., de Los Santos, T., Szczepanek, S.M., 2019. The VP1 G-H loop hypervariable epitope contributes to protective immunity against Foot and Mouth Disease Virus in swine. Vaccine 37, 3435-3442.

Fernandez-Sainz, I., Medina, G.N., Ramirez-Medina, E., Koster, M.J., Grubman, M.J., de Los Santos, T., 2017. Adenovirus-vectored foot-and-mouth disease vaccine confers early and full protection against FMDV O1 Manisa in swine. Virology 502, 123-132.

Fowler, V., Robinson, L., Bankowski, B., Cox, S., Parida, S., Lawlor, C., Gibson, D., O'Brien, F., Ellefsen, B., Hannaman, D., Takamatsu, H.H., Barnett, P.V., 2012. A DNA vaccination regime including protein boost and electroporation protects cattle against foot-and-mouth disease. Antiviral Res 94, 25-34.

Fox, G., Parry, N.R., Barnett, P.V., McGinn, B., Rowlands, D.J., Brown, F., 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J Gen Virol 70 (Pt 3), 625-637.

Fracastoius, H., 1546. De sympathia et antipathia rerum liber unus. De contagione et contagiosis morbis et eorum curatione liber I, Venice Heirs of L. A. Junta.

Francis, M.J., Black, L., 1983. Antibody response in pig nasal fluid and serum following foot-and-mouth disease infection or vaccination. J Hyg (Lond) 91, 329-334.

Fry, E.E., Stuart, D.I., Rowlands, D.J., 2005. The structure of foot-and-mouth disease virus. Curr Top Microbiol Immunol 288, 71-101.

Ganges, L., Borrego, B., Fernandez-Pacheco, P., Revilla, C., Fernandez-Borges, N., Dominguez, J., Sobrino, F., Rodriguez, F., 2011. DNA immunization of pigs with foot-and-mouth disease virus minigenes: from partial protection to disease exacerbation. Virus Res 157, 121-125.

Gao, F.S., Fang, Q.M., Li, Y.G., Li, X.S., Hao, H.F., Xia, C., 2006. Reconstruction of a swine SLA-I protein complex and determination of binding nonameric peptides derived from the foot-and-mouth disease virus. Vet Immunol Immunopathol 113, 328-338.

Garcia-Arriaza, J., Manrubia, S.C., Toja, M., Domingo, E., Escarmis, C., 2004. Evolutionary transition toward defective RNAs that are infectious by complementation. J Virol 78, 11678-11685.

Garcia-Briones, M.M., Blanco, E., Chiva, C., Andreu, D., Ley, V., Sobrino, F., 2004. Immunogenicity and T cell recognition in swine of foot-and-mouth disease virus polymerase 3D. Virology 322, 264-275.

Garcia-Briones, M.M., Russell, G.C., Oliver, R.A., Tami, C., Taboga, O., Carrillo, E., Palma, E.L., Sobrino, F., Glass, E.J., 2000. Association of bovine DRB3 alleles with immune response to FMDV peptides and protection against viral challenge. Vaccine 19, 1167-1171.

Garcia-Valcarcel, M., Doel, T., Collen, T., Ryan, M., Parkhouse, R.M., 1996. Recognition of foot-and-mouth disease virus and its capsid protein VP1 by bovine peripheral T lymphocytes. J Gen Virol 77 (Pt 4), 727-735.

Gebauer, F., de la Torre, J.C., Gomes, I., Mateu, M.G., Barahona, H., Tiraboschi, B., Bergmann, I., de Mello, P.A., Domingo, E., 1988. Rapid selection of genetic and antigenic variants of foot-and-mouth disease virus during persistence in cattle. J Virol 62, 2041-2049.

Gerner, W., Denyer, M.S., Takamatsu, H.H., Wileman, T.E., Wiesmuller, K.H., Pfaff, E., Saalmuller, A., 2006. Identification of novel foot-and-mouth disease virus specific T-cell epitopes in c/c and d/d haplotype miniature swine. Virus Res 121, 223-228.

Gerner, W., Hammer, S.E., Wiesmuller, K.H., Saalmuller, A., 2009. Identification of major histocompatibility complex restriction and anchor residues of foot-and-mouth disease virus-derived bovine T-cell epitopes. J Virol 83, 4039-4050.

Gerner, W., Talker, S.C., Koinig, H.C., Sedlak, C., Mair, K.H., Saalmuller, A., 2015. Phenotypic and functional differentiation of porcine alphabeta T cells: current knowledge and available tools. Mol Immunol 66, 3-13.

Golde, W.T., Nfon, C.K., Toka, F.N., 2008. Immune evasion during foot-and-mouth disease virus infection of swine. Immunol Rev 225, 85-95.

Grant, C.F.J., Carr, B.V., Singanallur, N.B., Morris, J., Gubbins, S., Hudelet, P., Ilott, M., Charreyre, C., Vosloo, W., Charleston, B., 2016. The B-cell response to foot-and-mouth-disease virus in cattle following vaccination and live-virus challenge. J Gen Virol 97, 2201-2209.

Gray, D., 2002. A role for antigen in the maintenance of immunological memory. Nat Rev Immunol 2, 60-65.

Grazioli, S., Fallacara, F., Brocchi, E., 2013. Mapping of antigenic sites of foot-and-mouth disease virus serotype Asia 1 and relationships with sites described in other serotypes. J Gen Virol 94, 559-569.

Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin Microbiol Rev 17, 465-493.

Grubman, M.J., Lewis, S.A., Morgan, D.O., 1993. Protection of swine against foot-and-mouth disease with viral capsid proteins expressed in heterologous systems. Vaccine 11, 825-829.

Gulbahar, M.Y., Davis, W.C., Guvenc, T., Yarim, M., Parlak, U., Kabak, Y.B., 2007. Myocarditis associated with foot-and-mouth disease virus type O in lambs. Vet Pathol 44, 589-599.

Gulce Iz, S., Doskaya, M., Borrego, B., Rodriguez, F., Guruz, Y., Gurhan, I.D., 2013. Co-expression of the Bcl-xL antiapoptotic protein enhances the induction of Th1-like immune responses in mice immunized with DNA vaccines encoding FMDV B and T cell epitopes. Vet Res Commun 37, 187-196.

Gullberg, M., Lohse, L., Botner, A., McInerney, G.M., Burman, A., Jackson, T., Polacek, C., Belsham, G.J., 2016. A Prime-Boost Vaccination Strategy in Cattle to Prevent Foot-and-Mouth Disease Using a "Single-Cycle" Alphavirus Vector and Empty Capsid Particles. PLoS One 11, e0157435.

Gullberg, M., Muszynski, B., Organtini, L.J., Ashley, R.E., Hafenstein, S.L., Belsham, G.J., Polacek, C., 2013. Assembly and characterization of foot-and-mouth disease virus empty capsid particles expressed within mammalian cells. J Gen Virol 94, 1769-1779.

Guo, H.C., Sun, S.Q., Jin, Y., Yang, S.L., Wei, Y.Q., Sun, D.H., Yin, S.H., Ma, J.W., Liu, Z.X., Guo, J.H., Luo, J.X., Yin, H., Liu, X.T., Liu, D.X., 2013. Foot-and-mouth disease virus-like particles produced by a SUMO fusion protein system in Escherichia coli induce potent protective immune responses in guinea pigs, swine and cattle. Vet Res 44, 48.

Guzman, E., Taylor, G., Charleston, B., Ellis, S.A., 2010. Induction of a cross-reactive CD8(+) T cell response following foot-and-mouth disease virus vaccination. J Virol 84, 12375-12384.

Guzman, E., Taylor, G., Charleston, B., Skinner, M.A., Ellis, S.A., 2008. An MHC-restricted CD8+ T-cell response is induced in cattle by foot-and-mouth disease virus (FMDV) infection and also following vaccination with inactivated FMDV. J Gen Virol 89, 667-675.

Habiela, M., Seago, J., Perez-Martin, E., Waters, R., Windsor, M., Salguero, F.J., Wood, J., Charleston, B., Juleff, N., 2014. Laboratory animal models to study foot-and-mouth disease: a review with emphasis on natural and vaccine-induced immunity. J Gen Virol 95, 2329-2345.

Han, S.C., Guo, H.C., Sun, S.Q., 2015. Three-dimensional structure of foot-and-mouth disease virus and its biological functions. Arch Virol 160, 1-16.

Han, S.C., Guo, H.C., Sun, S.Q., Jin, Y., Wei, Y.Q., Feng, X., Yao, X.P., Cao, S.Z., Xiang Liu, D., Liu, X.T., 2016. Productive Entry of Foot-and-Mouth Disease Virus via Macropinocytosis Independent of Phosphatidylinositol 3-Kinase. Sci Rep 6, 19294.

Hangartner, L., Zinkernagel, R.M., Hengartner, H., 2006. Antiviral antibody responses: the two extremes of a wide spectrum. Nat Rev Immunol 6, 231-243.

Hedger, R.S., Condy, J.B., 1985. Transmission of foot-and-mouth disease from African buffalo virus carriers to bovines. Vet Rec 117, 205.

Heegaard, P.M., Boas, U., Sorensen, N.S., 2010. Dendrimers for vaccine and immunostimulatory uses. A review. Bioconjug Chem 21, 405-418.

Hynes, R.O., 1987. Integrins: a family of cell surface receptors. Cell 48, 549-554.

Jackson, T., Ellard, F.M., Ghazaleh, R.A., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W., King, A.M., 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. J Virol 70, 5282-5287.

Jackson, T., King, A.M., Stuart, D.I., Fry, E., 2003. Structure and receptor binding. Virus Res 91, 33-46.

Jackson, T., Sheppard, D., Denyer, M., Blakemore, W., King, A.M., 2000. The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. J Virol 74, 4949-4956.

Juleff, N., Windsor, M., Lefevre, E.A., Gubbins, S., Hamblin, P., Reid, E., McLaughlin, K., Beverley, P.C., Morrison, I.W., Charleston, B., 2009. Foot-and-mouth disease virus can induce a specific and rapid CD4+ T-cell-independent neutralizing and isotype class-switched antibody response in naive cattle. J Virol 83, 3626-3636.

King, A.M., Underwood, B.O., McCahon, D., Newman, J.W., Brown, F., 1981. Biochemical identification of viruses causing the 1981 outbreaks of foot and mouth disease in the UK. Nature 293, 479-480.

Kitching, R.P., 1998. A recent history of foot-and-mouth disease. J Comp Pathol 118, 89-108.

Kitson, J.D., McCahon, D., Belsham, G.J., 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot and mouth disease virus: evidence for the involvement of the three surface exposed capsid proteins in four antigenic sites. Virology 179, 26-34.

Kleid, D.G., Yansura, D., Small, B., Dowbenko, D., Moore, D.M., Grubman, M.J., McKercher, P.D., Morgan, D.O., Robertson, B.H., Bachrach, H.L., 1981. Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. Science 214, 1125-1129.

Knowles, N.J., Samuel, A.R., 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Res 91, 65-80.

Knowles, N.J., Samuel, A.R., Davies, P.R., Midgley, R.J., Valarcher, J.F., 2005. Pandemic strain of foot-and-mouth disease virus serotype O. Emerg Infect Dis 11, 1887-1893.

Koff, W.C., Burton, D.R., Johnson, P.R., Walker, B.D., King, C.R., Nabel, G.J., Ahmed, R., Bhan, M.K., Plotkin, S.A., 2013. Accelerating next-generation vaccine development for global disease prevention. Science 340, 1232910.

Kotecha, A., Seago, J., Scott, K., Burman, A., Loureiro, S., Ren, J., Porta, C., Ginn, H.M., Jackson, T., Perez-Martin, E., Siebert, C.A., Paul, G., Huiskonen, J.T., Jones, I.M., Esnouf, R.M., Fry, E.E., Maree, F.F., Charleston, B., Stuart, D.I., 2015. Structure-based energetics of protein interfaces guides foot-and-mouth disease virus vaccine design. Nat Struct Mol Biol 22, 788-794.

Kotecha, A., Wang, Q., Dong, X., Ilca, S.L., Ondiviela, M., Zihe, R., Seago, J., Charleston, B., Fry, E.E., Abrescia, N.G.A., Springer, T.A., Huiskonen, J.T., Stuart, D.I., 2017. Rules of engagement between alphavbeta6 integrin and foot-and-mouth disease virus. Nat Commun 8, 15408.

Kotla, S., Sanghratna Vishanath, B., H, J.D., K, G., V, V.S.S., Reddy, G.R., 2016. DNA vaccine (P1-2A-3C-pCDNA) co-administered with Bovine IL-18 gives protective immune response against Foot and Mouth Disease in cattle. Vet Microbiol 193, 106-115.

Kunwar, P., Hawkins, N., Dinges, W.L., Liu, Y., Gabriel, E.E., Swan, D.A., Stevens, C.E., Maenza, J., Collier, A.C., Mullins, J.I., Hertz, T., Yu, X., Horton, H., 2013. Superior control of HIV-1 replication by CD8+ T cells targeting conserved epitopes: implications for HIV vaccine design. PLoS One 8, e64405.

Lamonaca, V., Missale, G., Urbani, S., Pilli, M., Boni, C., Mori, C., Sette, A., Massari, M., Southwood, S., Bertoni, R., Valli, A., Fiaccadori, F., Ferrari, C., 1999. Conserved hepatitis C virus sequences are highly immunogenic for CD4(+) T cells: implications for vaccine development. Hepatology 30, 1088-1098.

Lawrence, P., Rieder, E. 2017. Foot-and-mouth Disease Virus Receptors: Multiple Gateways to Initiate Infection, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus. Current Research and Emerging Trends. Caister Academic Press, Norfolk, UK, 107-136.

Lea, S., Hernandez, J., Blakemore, W., Brocchi, E., Curry, S., Domingo, E., Fry, E., Abu-Ghazaleh, R., King, A., Newman, J., et al., 1994. The structure and antigenicity of a type C foot-and-mouth disease virus. Structure 2, 123-139.

Lee, H.B., Piao, D.C., Lee, J.Y., Choi, J.Y., Bok, J.D., Cho, C.S., Kang, S.K., Choi, Y.J., 2017. Artificially designed recombinant protein composed of multiple epitopes of foot-and-mouth disease virus as a vaccine candidate. Microb Cell Fact 16, 33.

Lei, Y., Shao, J., Zhao, F., Li, Y., Lei, C., Ma, F., Chang, H., Zhang, Y., 2019. Artificially designed hepatitis B virus core particles composed of multiple epitopes of type A and O foot-and-mouth disease virus as a bivalent vaccine candidate. J Med Virol 91, 2142-2152.

Lennerz, V., Gross, S., Gallerani, E., Sessa, C., Mach, N., Boehm, S., Hess, D., von Boehmer, L., Knuth, A., Ochsenbein, A.F., Gnad-Vogt, U., Zieschang, J., Forssmann, U., Woelfel, T., Kaempgen, E., 2014. Immunologic response to the survivin-derived multi-epitope vaccine EMD640744 in patients with advanced solid tumors. Cancer Immunol Immunother 63, 381-394.

Li, W., Joshi, M.D., Singhania, S., Ramsey, K.H., Murthy, A.K., 2014. Peptide Vaccine: Progress and Challenges. Vaccines (Basel) 2, 515-536.

Liao, Y.C., Lin, H.H., Lin, C.H., Chung, W.B., 2013. Identification of cytotoxic T lymphocyte epitopes on swine viruses: multi-epitope design for universal T cell vaccine. PLoS One 8, e84443.

Liu, W., Yang, B., Wang, M., Wang, H., Yang, D., Ma, W., Zhou, G., Yu, L., 2017. Identification of a conformational neutralizing epitope on the VP1 protein of type A foot-and-mouth disease virus. Res Vet Sci 115, 374-381.

Liu, X.S., Wang, Y.L., Zhang, Y.G., Fang, Y.Z., Pan, L., Lu, J.L., Zhou, P., Zhang, Z.W., Jiang, S.T., 2011. Identification of H-2d restricted T cell epitope of foot-and-mouth disease virus structural protein VP1. Virol J 8, 426.

Loeffler, F., Frosch, P., 1897. Summarischer Bericht uber die Ergebnisse der Untersuchungen zur Erforschung der Maul- und Klauenseuche. Zentbl. Bakteriol. Parasitenkd Abt. I 22, 257-259.

Lyons, N.A., Knight-Jones, T.J.D., Bartels, C., Paton, D.J., Ferrari, G., Vermillion, M.S., Brooks, A.W., Motroni, R., Parker, E., Hefferin Berquist, M.L., Sumption, K.J., Klement, E., 2019. Considerations for design and implementation of vaccine field trials for novel foot-and-mouth disease vaccines. Vaccine 37, 1007-1015.

Mahapatra, M., Upadhyaya, S., Parida, S., 2019. Identification of novel epitopes in serotype O foot-and-mouth disease virus by in vitro immune selection. J Gen Virol 100, 804-811.

Malik, N., Kotecha, A., Gold, S., Asfor, A., Ren, J., Huiskonen, J.T., Tuthill, T.J., Fry, E.E., Stuart, D.I., 2017. Structures of foot and mouth disease virus pentamers: Insight into capsid dissociation and unexpected pentamer reassociation. PLoS Pathog 13, e1006607.

Marrack, P., Scott-Browne, J.P., Dai, S., Gapin, L., Kappler, J.W., 2008. Evolutionarily conserved amino acids that control TCR-MHC interaction. Annu Rev Immunol 26, 171-203.

Martin-Acebes, M.A., 2009. Mecanismos de entrada y de organización del complejo de replicación del virus de la fiebre aftosa: estudio comparativo con el virus de la enfermedad vesicular del cerdo y el virus de la estomatitis vesicular. Universidad Autonoma de Madrid, Madrid.

Mason, P.W., Grubman, M.J., Baxt, B., 2003. Molecular basis of pathogenesis of FMDV. Virus Res 91, 9-32.

Mason, P.W., Piccone, M.E., McKenna, T.S., Chinsangaram, J., Grubman, M.J., 1997. Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. Virology 227, 96-102.

Mateo, R., Diaz, A., Baranowski, E., Mateu, M.G., 2003. Complete alanine scanning of intersubunit interfaces in a foot-and-mouth disease virus capsid reveals critical contributions of many side chains to particle stability and viral function. J Biol Chem 278, 41019-41027.

Mateu, M.G., 1995. Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res 38, 1-24.

Mateu, M.G. 2017. The Foot-and-mouth Disease Virion: Structure and Function, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus. Current Research and Emerging Trends. Caister Academic Press, Norfolk, UK, 61-105.

Mateu, M.G., Escarmis, C., Domingo, E., 1998. Mutational analysis of discontinuous epitopes of foot-and-mouth disease virus using an unprocessed capsid protomer precursor. Virus Res 53, 27-37.

Mateu, M.G., Hernandez, J., Martinez, M.A., Feigelstock, D., Lea, S., Perez, J.J., Giralt, E., Stuart, D., Palma, E.L., Domingo, E., 1994. Antigenic heterogeneity of a foot-and-mouth disease virus serotype in the field is mediated by very limited sequence variation at several antigenic sites. J Virol 68, 1407-1417.

Mateu, M.G., Martinez, M.A., Capucci, L., Andreu, D., Giralt, E., Sobrino, F., Brocchi, E., Domingo, E., 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. J Gen Virol 71 (Pt 3), 629-637.

Mateu, M.G., Valero, M.L., Andreu, D., Domingo, E., 1996. Systematic replacement of amino acid residues within an Arg-Gly-Asp-containing loop of foot-and-mouth disease virus and effect on cell recognition. J Biol Chem 271, 12814-12819.

Mateu, M.G., Verdaguer, N. 2004. Functional and structural aspects of the interaction of foot-and-mouth disease virus with antibodies, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease. Current Perspectives. Horizon Bioscience, Wymondham, UK, 223-260.

Mattion, N., Konig, G., Seki, C., Smitsaart, E., Maradei, E., Robiolo, B., Duffy, S., Leon, E., Piccone, M., Sadir, A., Bottini, R., Cosentino, B., Falczuk, A., Maresca, R., Periolo, O., Bellinzoni, R., Espinoza, A., Torre, J.L., Palma, E.L., 2004. Reintroduction of foot-and-mouth disease in Argentina: characterisation of the isolates and development of tools for the control and eradication of the disease. Vaccine 22, 4149-4162.

Mayr, G.A., Chinsangaram, J., Grubman, M.J., 1999. Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. Virology 263, 496-506.

McCullough, K., Saiz, M., Summerfield, A. 2017. Innate to Adaptive: Immune Defense Handling of Foot-and-mouth Disease Virus, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus. Current Research and Emerging Trends. Caister Academic Press, Norfolk, UK, 211-274.

McCullough, K., Sobrino, F. 2004. Immunology of Foot-and-Mouth Disease, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-Mouth Disease. Current Perspectives. Horizon Bioscience, Norfolk, UK, 173-222.

McCullough, K.C., Bruckner, L., Schaffner, R., Fraefel, W., Muller, H.K., Kihm, U., 1992a. Relationship between the anti-FMD virus antibody reaction as measured by different assays, and protection in vivo against challenge infection. Vet Microbiol 30, 99-112.

McCullough, K.C., De Simone, F., Brocchi, E., Capucci, L., Crowther, J.R., Kihm, U., 1992b. Protective immune response against foot-and-mouth disease. J Virol 66, 1835-1840.

McCullough, K.C., Parkinson, D., Crowther, J.R., 1988. Opsonization-enhanced phagocytosis of foot-and-mouth disease virus. Immunology 65, 187-191.

McKenna, T.S., Lubroth, J., Rieder, E., Baxt, B., Mason, P.W., 1995. Receptor binding site-deleted foot-and-mouth disease (FMD) virus protects cattle from FMD. J Virol 69, 5787-5790.

McVicar, J.W., Richmond, J.Y., Campbell, C.H., Hamilton, L.D., 1973. Observations of cattle, goats and pigs after administration of synthetic interferon inducers and subsequent exposure to foot and mouth disease virus. Can J Comp Med 37, 362-368.

Monso, M., de la Torre, B.G., Blanco, E., Moreno, N., Andreu, D., 2013. Influence of conjugation chemistry and B epitope orientation on the immune response of branched peptide antigens. Bioconjug Chem 24, 578-585.

Moonen, P., Schrijver, R., 2000. Carriers of foot-and-mouth disease virus: a review. Vet Q 22, 193-197.

Moraes, M.P., Chinsangaram, J., Brum, M.C., Grubman, M.J., 2003. Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit vaccine. Vaccine 22, 268-279.

Moraes, M.P., Mayr, G.A., Mason, P.W., Grubman, M.J., 2002. Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. Vaccine 20, 1631-1639.

Mowat, G.N., Chapman, W.G., 1962. Growth of foot-and-mouth disease virus in a fibroblastic cell line derived from hamster kidneys. Nature 194, 253-255.

Mueller, S., Papamichail, D., Coleman, J.R., Skiena, S., Wimmer, E., 2006. Reduction of the rate of poliovirus protein synthesis through large-scale codon deoptimization causes attenuation of viral virulence by lowering specific infectivity. J Virol 80, 9687-9696.

Mulcahy, G., Gale, C., Robertson, P., Iyisan, S., DiMarchi, R.D., Doel, T.R., 1990. Isotype responses of infected, virus-vaccinated and peptide-vaccinated cattle to foot-and-mouth disease virus. Vaccine 8, 249-256.

Naessens, J., Scheerlinck, J.P., De Buysscher, E.V., Kennedy, D., Sileghem, M., 1998. Effective in vivo depletion of T cell subpopulations and loss of memory cells in cattle using mouse monoclonal antibodies. Vet Immunol Immunopathol 64, 219-234.

Nfon, C., Lung, O., Embury-Hyatt, C., Alexandersen, S. 2017. Clinical Signs and Pathology of Foot-and-Mouth Disease In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus Current Research and Emerging Trends. Caister Academic Press Norfolk, UK, 171-178.

Nunez, J.I., Molina, N., Baranowski, E., Domingo, E., Clark, S., Burman, A., Berryman, S., Jackson, T., Sobrino, F., 2007. Guinea pig-adapted foot-and-mouth disease virus with altered receptor recognition can productively infect a natural host. J Virol 81, 8497-8506.

Oh, Y., Fleming, L., Statham, B., Hamblin, P., Barnett, P., Paton, D.J., Park, J.H., Joo, Y.S., Parida, S., 2012. Interferon-gamma induced by in vitro re-stimulation of CD4+ T-cells correlates with in vivo FMD vaccine induced protection of cattle against disease and persistent infection. PLoS One 7, e44365.

OIE, 2018. Manual of Diagnostic Tests and Vaccines for Tesrrestrial Animals, 8 EditionParis, France.

Opperman, P.A., Maree, F.F., Van Wyngaardt, W., Vosloo, W., Theron, J., 2012. Mapping of antigenic determinants on a SAT2 foot-and-mouth disease virus using chicken single-chain antibody fragments. Virus Res 167, 370-379.

Pacheco, J.M., Brum, M.C., Moraes, M.P., Golde, W.T., Grubman, M.J., 2005. Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. Virology 337, 205-209.

Parida, S., 2009. Vaccination against foot-and-mouth disease virus: strategies and effectiveness. Expert Rev Vaccines 8, 347-365.

Parry, N., Fox, G., Rowlands, D., Brown, F., Fry, E., Acharya, R., Logan, D., Stuart, D., 1990. Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. Nature 347, 569-572.

Parry, N.R., Ouldridge, E.J., Barnett, P.V., Clarke, B.E., Francis, M.J., Fox, J.D., Rowlands, D.J., Brown, F., 1989. Serological prospects for peptide vaccines against foot-and-mouth disease virus. J Gen Virol 70 (Pt 11), 2919-2930.

Patch, J.R., Pedersen, L.E., Toka, F.N., Moraes, M., Grubman, M.J., Nielsen, M., Jungersen, G., Buus, S., Golde, W.T., 2011. Induction of foot-and-mouth disease virus-specific cytotoxic T cell killing by vaccination. Clin Vaccine Immunol 18, 280-288.

Pereda, A.J., Konig, G.A., Chimeno Zoth, S.A., Borca, M., Palma, E.L., Piccone, M.E., 2002. Full length nucleotide sequence of foot-and-mouth disease virus strain O1 Campos/Bra/58. Brief report. Arch Virol 147, 2225-2230.

Pereira, H.G., 1981. Virus Diseases of Foof Animals, Vol 2. Academic Press, London.

Perez, A.M., Willeberg, P.W., 2017. Editorial: Foot-and-Mouth Disease in Swine. Front Vet Sci 4, 133.

Perez Filgueira, D.M., Berinstein, A., Smitsaart, E., Borca, M.V., Sadir, A.M., 1995. Isotype profiles induced in Balb/c mice during foot and mouth disease (FMD) virus infection or immunization with different FMD vaccine formulations. Vaccine 13, 953-960.

Pfaff, E., Mussgay, M., Bohm, H.O., Schulz, G.E., Schaller, H., 1982. Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. EMBO J 1, 869-874.

Pintaric, M., Gerner, W., Saalmuller, A., 2008. Synergistic effects of IL-2, IL-12 and IL-18 on cytolytic activity, perforin expression and IFN-gamma production of porcine natural killer cells. Vet Immunol Immunopathol 121, 68-82.

Porta, C., Xu, X., Loureiro, S., Paramasivam, S., Ren, J., Al-Khalil, T., Burman, A., Jackson, T., Belsham, G.J., Curry, S., Lomonossoff, G.P., Parida, S., Paton, D., Li, Y., Wilsden, G., Ferris, N., Owens, R., Kotecha, A., Fry, E., Stuart, D.I., Charleston, B., Jones, I.M., 2013. Efficient production of foot-and-mouth disease virus empty capsids in insect cells following down regulation of 3C protease activity. J Virol Methods 187, 406-412.

Rai, D.K., Diaz-San Segundo, F., Campagnola, G., Keith, A., Schafer, E.A., Kloc, A., de Los Santos, T., Peersen, O., Rieder, E., 2017. Attenuation of Foot-and-Mouth Disease Virus by Engineered Viral Polymerase Fidelity. J Virol 91.

Ramesh, S., Cherkupally, P., Govender, T., Kruger, H.G., Albericio, F., de la Torre, B.G., 2015. Chemical Platforms for Peptide Vaccine Constructs. Adv Protein Chem Struct Biol 99, 99-130.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., Jenkins, M.K., 2001. Visualizing the generation of memory CD4 T cells in the whole body. Nature 410, 101-105.

Reutner, K., Leitner, J., Essler, S.E., Witter, K., Patzl, M., Steinberger, P., Saalmuller, A., Gerner, W., 2012. Porcine CD27: identification, expression and functional aspects in lymphocyte subsets in swine. Dev Comp Immunol 38, 321-331.

Revilla, C., Chamorro, S., Alvarez, B., Perez, C., Ezquerra, A., Alonso, F., Dominguez, J., 2005. Analysis of functional heterogeneity of porcine memory CD4+ T cells. Dev Comp Immunol 29, 479-488.

Revilla, C., Rodriguez-Carreno, M.P., Alvarez, B., Chamorro, S., Alonso, L.M., Ezquerra, A., Alonso, F., Dominguez, J., 2004. 2E3, a new marker that selectively identifies porcine CD4+ naive T cells. Dev Comp Immunol 28, 239-250.

Robinson, L., Knight-Jones, T.J., Charleston, B., Rodriguez, L.L., Gay, C.G., Sumption, K.J., Vosloo, W., 2016. Global Foot-and-Mouth Disease Research Update and Gap Analysis: 6 - Immunology. Transbound Emerg Dis 63 Suppl 1, 56-62.

Rodriguez-Carreno, M.P., Lopez-Fuertes, L., Revilla, C., Ezquerra, A., Alonso, F., Dominguez, J., 2002. Phenotypic characterization of porcine IFN-gamma-producing lymphocytes by flow cytometry. J Immunol Methods 259, 171-179.

Rodriguez, A., Ley, V., Ortuno, E., Ezquerra, A., Saalmuller, A., Sobrino, F., Saiz, J.C., 1996. A porcine CD8+ T cell clone with heterotypic specificity for foot-and-mouth disease virus. J Gen Virol 77 (Pt 9), 2089-2096.

Rodriguez, A., Saiz, J.C., Novella, I.S., Andreu, D., Sobrino, F., 1994. Antigenic specificity of porcine T cell response against foot-and-mouth disease virus structural proteins: identification of T helper epitopes in VP1. Virology 205, 24-33.

Rodriguez, L.L., Barrera, J., Kramer, E., Lubroth, J., Brown, F., Golde, W.T., 2003. A synthetic peptide containing the consensus sequence of the G-H loop region of foot-and-mouth disease virus type-O VP1 and a promiscuous T-helper epitope induces peptide-specific antibodies but fails to protect cattle against viral challenge. Vaccine 21, 3751-3756.

Rodriguez, L.L., Grubman, M.J., 2009. Foot and mouth disease virus vaccines. Vaccine 27 Suppl 4, D90-94.

Roosien, J., Belsham, G.J., Ryan, M.D., King, A.M., Vlak, J.M., 1990. Synthesis of foot-and-mouth disease virus capsid proteins in insect cells using baculovirus expression vectors. J Gen Virol 71 (Pt 8), 1703-1711.

Rosendahl Huber, S., van Beek, J., de Jonge, J., Luytjes, W., van Baarle, D., 2014. T cell responses to viral infections - opportunities for Peptide vaccination. Front Immunol 5, 171.

Sa-Carvalho, D., Rieder, E., Baxt, B., Rodarte, R., Tanuri, A., Mason, P.W., 1997. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J Virol 71, 5115-5123.

Saiz, J.C., Rodriguez, A., Gonzalez, M., Alonso, F., Sobrino, F., 1992. Heterotypic lymphoproliferative response in pigs vaccinated with foot-and-mouth disease virus. Involvement of isolated capsid proteins. J Gen Virol 73 (Pt 10), 2601-2607.

Saiz, M., De La Morena, D.B., Blanco, E., Nunez, J.I., Fernandez, R., Sanchez-Vizcaino, J.M., 2003. Detection of foot-and-mouth disease virus from culture and clinical samples by reverse transcription-PCR coupled to restriction enzyme and sequence analysis. Vet Res 34, 105-117.

Saiz, M., Gomez, S., Martinez-Salas, E., Sobrino, F., 2001. Deletion or substitution of the aphthovirus 3' NCR abrogates infectivity and virus replication. J Gen Virol 82, 93-101.

Salguero, F.J., Sanchez-Martin, M.A., Diaz-San Segundo, F., de Avila, A., Sevilla, N., 2005. Foot-and-mouth disease virus (FMDV) causes an acute disease that can be lethal for adult laboratory mice. Virology 332, 384-396.

Salt, J.S., 1993. The carrier state in foot and mouth disease--an immunological review. Br Vet J 149, 207-223.

Salt, J.S., Barnett, P.V., Dani, P., Williams, L., 1998. Emergency vaccination of pigs against foot-and-mouth disease: protection against disease and reduction in contact transmission. Vaccine 16, 746-754.

Salt, J.S., Mulcahy, G., Kitching, R.P., 1996. Isotype-specific antibody responses to foot-and-mouth disease virus in sera and secretions of "carrier' and "non-carrier' cattle. Epidemiol Infect 117, 349-360.

Sallusto, F., Lanzavecchia, A., 2009. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. Eur J Immunol 39, 2076-2082.

Sallusto, F., Lanzavecchia, A., Araki, K., Ahmed, R., 2010. From vaccines to memory and back. Immunity 33, 451-463.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., Lanzavecchia, A., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401, 708-712.

Sanz-Parra, A., Jimenez-Clavero, M.A., Garcia-Briones, M.M., Blanco, E., Sobrino, F., Ley, V., 1999. Recombinant viruses expressing the foot-and-mouth disease virus capsid precursor polypeptide (P1) induce cellular but not humoral antiviral immunity and partial protection in pigs. Virology 259, 129-134.

Sanz-Parra, A., Sobrino, F., Ley, V., 1998. Infection with foot-and-mouth disease virus results in a rapid reduction of MHC class I surface expression. J Gen Virol 79 (Pt 3), 433-436.

Scott, C.A., Peterson, P.A., Teyton, L., Wilson, I.A., 1998. Crystal structures of two I-Ad-peptide complexes reveal that high affinity can be achieved without large anchor residues. Immunity 8, 319-329.

Schutta, C., Barrera, J., Pisano, M., Zsak, L., Grubman, M.J., Mayr, G.A., Moraes, M.P., Kamicker, B.J., Brake, D.A., Ettyreddy, D., Brough, D.E., Butman, B.T., Neilan, J.G., 2016. Multiple efficacy studies of an adenovirus-vectored foot-and-mouth disease virus serotype A24 subunit vaccine in cattle using homologous challenge. Vaccine 34, 3214-3220.

Segundo, F.D., Weiss, M., Perez-Martin, E., Dias, C.C., Grubman, M.J., Santos Tde, L., 2012. Inoculation of swine with foot-and-mouth disease SAP-mutant virus induces early protection against disease. J Virol 86, 1316-1327.

Sellers, R., Gloster, J., 2008. Foot-and-mouth disease: a review of intranasal infection of cattle, sheep and pigs. Vet J 177, 159-168.

Sitt, T., Kenney, M., Barrera, J., Pandya, M., Eckstrom, K., Warner, M., Pacheco, J.M., LaRocco, M., Palarea-Albaladejo, J., Brake, D., Rieder, E., Arzt, J., Barlow, J.W., Golde, W.T., 2019. Duration of protection and humoral immunity induced by an adenovirus-vectored subunit vaccine for foot-and-mouth disease (FMD) in Holstein steers. Vaccine 37, 6221-6231.

Smitsaart, E., Bergmann, I. 2017. Quality Attributes of Current Inactivated Foot-and-mouth Disease Vaccines and their Effects on the Success of Vaccination, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus. Current Research and Emerging Trends. Caister Academic Press, Norfolk, UK, 287-316.

Sobrino, F., Blanco, E., Garcia-Briones, M., Ley, V., 1999. Synthetic peptide vaccines: foot-and-mouth disease virus as a model. Dev Biol Stand 101, 39-43.

Sobrino, F., Davila, M., Ortin, J., Domingo, E., 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell culture. Virology 128, 310-318.

Sobrino, F., Domingo, E. 2001. Foot-and-mouth disease in Europe. FMD is economically the most important disease of farm animals. Its re-emergence in Europe is likely to have

consequences that go beyond severe alterations of livestock production and trade, Rep, E., ed., 459-561.

Sobrino, F., Saiz, M., Jimenez-Clavero, M.A., Nunez, J.I., Rosas, M.F., Baranowski, E., Ley, V., 2001. Foot-and-mouth disease virus: a long known virus, but a current threat. Vet Res 32, 1-30.

Soria, I., Quattrocchi, V., Langellotti, C., Gammella, M., Digiacomo, S., Garcia de la Torre, B., Andreu, D., Montoya, M., Sobrino, F., Blanco, E., Zamorano, P., 2017. Dendrimeric peptides can confer protection against foot-and-mouth disease virus in cattle. PLoS One 12, e0185184.

Soria, I., Quattrocchi, V., Langellotti, C., Perez-Filgueira, M., Pega, J., Gnazzo, V., Romera, S., Schammas, J., Bucafusco, D., Di Giacomo, S., de la Torre, B.G., Andreu, D., Sobrino, F., Blanco, E., Zamorano, P., 2018. Immune Response and Partial Protection against Heterologous Foot-and-Mouth Disease Virus Induced by Dendrimer Peptides in Cattle. J Immunol Res 2018, 3497401.

Steigerwald, R., Brake, D.A., Barrera, J., Schutta, C.J., Kalla, M., Wennier, S.T., Volkmann, A., Hurtle, W., Clark, B.A., Zurita, M., Pisano, M., Kamicker, B.J., Puckette, M.C., Rasmussen, M.V., Neilan, J.G., 2019. Evaluation of modified Vaccinia Ankara-based vaccines against foot-and-mouth disease serotype A24 in cattle. Vaccine.

Stenfeldt, C., Eschbaumer, M., Rekant, S.I., Pacheco, J.M., Smoliga, G.R., Hartwig, E.J., Rodriguez, L.L., Arzt, J., 2016. The Foot-and-Mouth Disease Carrier State Divergence in Cattle. J Virol 90, 6344-6364.

Stenfeldt, C., Eschbaumer, M., Smoliga, G.R., Rodriguez, L.L., Zhu, J., Arzt, J., 2017. Clearance of a persistent picornavirus infection is associated with enhanced pro-apoptotic and cellular immune responses. Sci Rep 7, 17800.

Stoker, M., Macpherson, I., 1964. Syrian Hamster Fibroblast Cell Line Bhk21 and Its Derivatives. Nature 203, 1355-1357.

Strohmaier, K., Franze, R., Adam, K.H., 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. J Gen Virol 59, 295-306.

Sutmoller, P., Gaggero, A., 1965. Foot-and mouth diseases carriers. Vet Rec 77, 968-969.

Taboga, O., Tami, C., Carrillo, E., Nunez, J.I., Rodriguez, A., Saiz, J.C., Blanco, E., Valero, M.L., Roig, X., Camarero, J.A., Andreu, D., Mateu, M.G., Giralt, E., Domingo, E., Sobrino, F., Palma, E.L., 1997. A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. J Virol 71, 2606-2614.

Talker, S.C., Kaser, T., Reutner, K., Sedlak, C., Mair, K.H., Koinig, H., Graage, R., Viehmann, M., Klingler, E., Ladinig, A., Ritzmann, M., Saalmuller, A., Gerner, W., 2013. Phenotypic maturation of porcine NK- and T-cell subsets. Dev Comp Immunol 40, 51-68.

Talker, S.C., Koinig, H.C., Stadler, M., Graage, R., Klingler, E., Ladinig, A., Mair, K.H., Hammer, S.E., Weissenbock, H., Durrwald, R., Ritzmann, M., Saalmuller, A., Gerner, W., 2015. Magnitude and kinetics of multifunctional CD4+ and CD8beta+ T cells in pigs infected with swine influenza A virus. Vet Res 46, 52.

Tam, J.P., 1988. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci U S A 85, 5409-5413.

Toja, M., Escarmis, C., Domingo, E., 1999. Genomic nucleotide sequence of a foot-and-mouth disease virus clone and its persistent derivatives. Implications for the evolution of viral quasispecies during a persistent infection. Virus Res 64, 161-171.

Tsuji, M., Zavala, F., 2001. Peptide-based subunit vaccines against pre-erythrocytic stages of malaria parasites. Mol Immunol 38, 433-442.

van Lierop, M.J., van Noort, J.M., Wagenaar, J.P., Rutten, V.P., Langeveld, J., Meloen, R.H., Hensen, E.J., 1994. T cell-stimulatory fragments of foot-and-mouth disease virus released by mild treatment with cathepsin D. J Gen Virol 75 (Pt 11), 2937-2946.

Verdaguer, N., Sevilla, N., Valero, M.L., Stuart, D., Brocchi, E., Andreu, D., Giralt, E., Domingo, E., Mateu, M.G., Fita, I., 1998. A similar pattern of interaction for different antibodies with

a major antigenic site of foot-and-mouth disease virus: implications for intratypic antigenic variation. J Virol 72, 739-748.

Villen, J., Borras, E., Schaaper, W.M., Meloen, R.H., Davila, M., Domingo, E., Giralt, E., Andreu, D., 2002. Functional mimicry of a discontinuous antigenic site by a designed synthetic peptide. Chembiochem 3, 175-182.

Villen, J., Rodriguez-Mias, R.A., Nunez, J.I., Giralt, E., Sobrino, F., Andreu, D., 2006. Rational dissection of binding surfaces for mimicking of discontinuous antigenic sites. Chem Biol 13, 815-823.

Vosloo, W., Thomson , G.R. 2017. Natural Habitats in which Foot-and-mouth Disease Viruses are Maintained, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus. Current Research and Emerging Trends. Caister Academic Press, Norfolk, UK, 179-210.

Wang, C.Y., Chang, T.Y., Walfield, A.M., Ye, J., Shen, M., Chen, S.P., Li, M.C., Lin, Y.L., Jong, M.H., Yang, P.C., Chyr, N., Kramer, E., Brown, F., 2002. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. Vaccine 20, 2603-2610.

Xiao, Y., Chen, H.Y., Wang, Y., Yin, B., Lv, C., Mo, X., Yan, H., Xuan, Y., Huang, Y., Pang, W., Li, X., Yuan, Y.A., Tian, K., 2016. Large-scale production of foot-and-mouth disease virus (serotype Asia1) VLP vaccine in Escherichia coli and protection potency evaluation in cattle. BMC Biotechnol 16, 56.

Zhang, L., 2018. Multi-epitope vaccines: a promising strategy against tumors and viral infections. Cell Mol Immunol 15, 182-184.

Zhang, Z., Pan, L., Ding, Y., Zhou, P., Lv, J., Chen, H., Fang, Y., Liu, X., Chang, H., Zhang, J., Shao, J., Lin, T., Zhao, F., Zhang, Y., Wang, Y., 2015. Efficacy of synthetic peptide candidate vaccines against serotype-A foot-and-mouth disease virus in cattle. Appl Microbiol Biotechnol 99, 1389-1398.

Zheng, M., Jin, N., Zhang, H., Jin, M., Lu, H., Ma, M., Li, C., Yin, G., Wang, R., Liu, Q., 2006. Construction and immunogenicity of a recombinant fowlpox virus containing the capsid and 3C protease coding regions of foot-and-mouth disease virus. J Virol Methods 136, 230-237.

Zinkernagel, R.M., 2003. On natural and artificial vaccinations. Annu Rev Immunol 21, 515-546.

Zinkernagel, R.M., 2018. What if protective immunity is antigen-driven and not due to so-called "memory" B and T cells? Immunol Rev 283, 238-246.

Zuckermann, F.A., Husmann, R.J., 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87, 500-512.